THE UNIVERSITY of EDINBURGH

Title	Cold shock response of Salmonella enterica serovar Typhimurium
Author	Holden, Nicola Jean.
Qualification	PhD
Year	2000

Digitisation Notes:

- Page 147 missing from original

THE COLD SHOCK RESPONSE OF *Salmonella enterica* serovar Typhimurium.

Nicola Jean Holden

Thesis presented for the degree of Doctor of Philosophy

University of Edinburgh

DECLARATION

I declare that this thesis was composed by myself and the research presented is my own.

Nicola Jean Holden

January 2000.

ACKNOWLEDGEMENTS

The thesis was supported by MAFF.

I would like to thank all the members of Maurice Gallagher's lab for their support and friendship throughout this study, especially Patrick Taylor, Moira Preistly and Teresa McBride who could always lighten up the darker moments. I would also like to thank Maurice Gallagher for his supervision, guidance and stimulating intellectual discussions.

This thesis would not have been possible without the unerring love and constant encouragement from Lez. I dedicate this thesis to him.

TABLE OF CONTENTS

TABLE OF TABLES

TABLE OF FIGURES

ABREVIATIONS

Amp^R	ampicillin resistant
bp	base pair
c.f.u.	colony forming units
Ci	Curies
Cml^R	chloramphenicol resistant
CTAB	hexadecyltrimethyl ammonium bromide
Da	Daltons
dCTP	deoxycytidine triphosphate
dH_2O	distilled water
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
dsDNA	double stranded DNA
dsRNA	double stranded RNA
DTT	dithiothreitrol
EDTA	ethylenediaminetetraacetate
g	grams
g	standard acceleration of gravity
IPTG	isoproyl-β-D-thiogalactoside
Kan^R	kanamycin resistant
kb	kilobases
l	litres
LB	Luria Bertani medium
M	molar
mA	milliamps
MAFF	Ministry of Agriculture, Fisheries and Food
mg	milligrams
ml	millilitres
µl	microlitres
mm	millimetres
mM	millimolar
MW	molecular weight
nt	nucleotide

°C	degrees Celsius
OD	optical density
ONPG	o-nitrophenyl-β-D-galactoside
ORF	open reading frame
PAGE	polyacrylamide gel electophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pI	isoelectric point
RBS	ribosome binding site
RNA	ribonucleic acid
SAP	shrimp alkaline phosphate
SD	Shine Delgarno
SDS	sodium dodecyl sulphate
SGSC	Salmonella Genetic Stock Centre
ssDNA	single stranded DNA
ssRNA	single stranded RNA
Tet^R	tetracyline resistant
TEMED	N,N,N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
US FDA	United States Food and Drug Administration
UV	ultraviolet
V	volts
v/v	volume per volume
w/v	weight per volume
WHO	World Health Organisation
X-gal	5-bromo-4-chloro-indoyl-β-D-galactoside
2-D PAGE	2-Dimensional PAGE

ABSTRACT.

The ability of *Salmonella enterica* serovar Tyhimurium (*S. typhimurium*) to adapt to and survive in refrigerated conditions plays a role in its capacity to cause food-borne disease. Exposure to low temperature elicits a classic stress response in many bacteria, a major feature of which is high level induction of CspA homologues. The exact role of these proteins at low temperatures is unknown. In the present study, mutation of *S. typhimurium cspA*, which encodes the major cold shock protein, was attempted using a variety of strategies. However, none of these resulted in recovery of mutants. This may have been due to the essential nature of *S. typhimurium cspA*, or alternatively, to transformation inefficiency.

Reporter studies showed that expression of a plasmid-based *S. typhimurium cspA-lacZ* fusion increased 8-fold over a period of 5 hours, when exponentially growing cells were shifted from 37°C to 15°C. This level of induction was lower than observed for the level of *S. typhimurium* CspA protein directly at 10°C and was less than that reported for similar studies with *E. coli cspA*. This discrepancy may indicate either the absence of a putative enhancer element, termed the downstream box, in the *lacZ* fusion construct or the absence of a chromosome-based *cis* acting element.

Regulation of expression of *S. typhimurium cspB* was examined at low temperatures using a bioluminescent reporter system. Expression of *cspB* was not detected at 30°C, but was highly induced at 10°C and to a lesser extent at 4°C, when the culture was in exponential phase. Expression of *cspB* was growth phase-dependent, such that bioluminescence from the *cspB*::Mu*dlux* fusion was approximately 10-fold lower at 10°C when the cells were in stationary phase cells, compared to an exponential phase culture. Expression of *cspB* at 4°C was minimal when the culture was in stationary phase. The alternative sigma factor, σ^s, did not appear to play a significant role in *cspB* expression at low temperature. In contrast, Fis appeared to act as a positive regulator of *cspB* in stationary phase cultures.

Cell survival assays (measured by ability to form colony forming units on nutrient agar plates) showed that 4 % of cells that were in early exponential phase survived a rapid cold shock to 4°C, although survival was almost complete when cells were in lag phase or in late exponential phase. The alternative sigma factor, σ^s, did not appear to play a significant role in cell survival in response to a rapid temperature reduction to 4°C. The addition of an osmoprotectant, 0.3 M sucrose, protected against loss of plating viability to some degree for early exponential phase cells, when diluted to 4°C.

2-D PAGE analysis showed that the response of exponential phase *S. typhimurium* cells incubated at 10°C consisted of an adaptive phase followed by an acclimation phase, in agreement with previous reports for *E. coli*. Identification of CspA was verified by N-terminal sequencing. The response was delayed at 4°C and recovery of protein synthesis in the acclimation phase was not as extensive, as observed at 10°C. CspA was synthesised throughout the period of incubation at 4°C. Growth phase was found to severely affect *de novo* protein synthesis at low temperature. Incubation of stationary phase cells at 10°C or 4°C resulted in repression of the synthesis of the majority of proteins, although a small set of proteins was induced. CspA was not detected at 37°C, but was highly induced at 10°C or 4°C. However, prolonged incubation at 4°C led to complete repression of protein synthesis, except for CspA.

This study has shown that *S. typhimurium* adapts to low temperature in a dynamic fashion and expression of CspA is a major feature of the response. Furthermore, it appears that exponential phase *S. typhimurium* cells are metabolically active even after 4 days at refrigeration temperatures.

CHAPTER 1

INTRODUCTION

1.1 Overview

Salmonella enterica is a major cause of food poisoning world-wide and is responsible for approximately 3 million deaths per annum, arising from acute gastro-enteritis/diarrhoea due to non-typhoidal salmonellosis (Pang *et al.*, 1995). An essential aspect that underpins the 'success' of this organism as a food-borne pathogen is the ability to survive in sub-optimal conditions such as refrigeration. Although survival studies of bacteria at low temperatures have been carried out as early as 1934 (Sharman & Cameron) and 1958 (Meynell), the molecular basis that underpins survival has only recently been examined (Jones *et al.*, 1987). This thesis aims to investigate the molecular aspects that enable an important food-borne pathogen to survive at low temperatures. This chapter is divided into three sections, which summarise food-borne illness, examines the basis for adaptive responses of bacteria to stressful environments and reviews what is known of how bacteria respond to incubation at low temperature, termed the cold shock response.

For the purpose of this thesis, *Salmonella enterica* serovar TYPHIMURIUM will be known as *S. typhimurium*.

1.2 Food Poisoning

1.2.1 The definition of food poisoning.

The term food poisoning is frequently connected, in the popular press, with outbreaks of food-borne illness brought about by bacterial pathogens. Strictly speaking, food poisoning refers to any 'foreign' agent encountered in food that provokes an allergic or toxic reaction. Food-borne illness can be brought about by 2 routes, either by toxicity or from infection. Toxicity may arise from chemical or biological sources. Chemical agents can be intrinsic to foods, for example toxic compounds found in non-edible fungi. Alternatively, extrinsic chemical poisoning may result from a wide range of sources such as accidental or environmental contamination. Biological toxicity also leads to food poisoning, for example, some food-associated moulds produce toxic metabolites, such as alfatoxins that have been associated with diseases

such as hepatitis, cirrhosis and Rey's syndrome. The second route to food poisoning is through infections from organisms, including bacteria, animal parasites, microfungi, viruses and protozoa, which are associated with the food. Bacterial infection arises from contamination of the food with specific pathogens, some of which also produce toxins when they have infected the host. Animal parasites may also be sources of food-borne illness. Such illnesses are brought about by the presence of these organisms in infected meat which is then consumed. Some viruses are associated with food-borne diseases such as gastro-enteritis and hepatitis, and some protozoa are associated with diarrhoea (Varnam & Evans, 1996).

1.2.2 The symptoms of food-borne illness.

The most widely known symptoms of food-borne illness are those of acute gastrointestinal illness and include diarrhoea (which may be bloody) and/or vomiting, together with the associated symptoms of abdominal pain and discomfort. However, some enteric pathogens can cause severe and sometimes life-threatening extraintestinal illnesses, such as typhoid fever caused by *Salmonella typhi* or HUS (haemolytic uraemic syndrome) caused by verotxin producing *Escherichia coli.* In addition, some food-borne pathogens, including *Salmonella* serovars, are also associated with reactive arthritis and autoimmune diseases (Archer & Young, 1988).

1.2.3 The scale of bacterial food poisoning.

On a global basis, food poisoning is a serious problem. In developed countries, such as the UK and the USA, it has been estimated that there are approximately 120 reported cases of food poisoning per 100,000 population (Maurice, 1994). However, the World Health Organisation (Press release WHO / 58, 1997) has suggested that as little as 3.0 – 3.5 % of food poisoning cases are reported. The situation is highly variable and the number of people infected from a single source of infection can range from one to several thousand. For example, in 1987 in the USA, as many as 15,000 cases of food-borne illnesses were due to consumption of milk infected with *Salmonella* (Ryan *et al.*, 1987). This situation is poorly reflected in terms of gross annual statistics.

The scale, efficiency and speed at which modern food production proceeds, can lead to a large number of cases that arise from a single source. To illustrate, in poultry plants which utilise a conveyor belt system, a single contaminated bird has the capacity to cross-contaminate a whole batch of birds during the evisceration stage, since the same equipment is used to process all the carcasses. Although steps such as reducing the carcass temperature to 4°C and washing with a dilute solution of hypochlorite are taken to reduce bacterial contamination following this type of procedure, the potential for wide-spread infection remains high.

1.2.4 The economic costs arising from bacterial associated food poisoning .

Overall, the economic costs arising from food poisoning are considerable. In addition to the medical costs and productivity losses, there are also industry costs arising from factors such as product recall and public loss of confidence in a particular product or reseller. The annual costs arising from *Salmonella* infections in the U.K., include the costs from (i) monitoring the incidence of salmonellae in agricultural livestock and animal foodstuffs, and (ii) taking the appropriate action to reduce the risk to public health. These alone total approximately £4.5M (MAFF, 1997). It has been estimated that in the UK, the annual costs arising from food poisoning exceeds £1b and in the United States of America, the annual costs are estimated at more than $7b (Economic Research Service, United States Department of Agriculture). Direct estimations of costs arising from a single outbreak are often difficult or impossible to make, but the following example illustrates the scale of the problem. It has been estimated that a single outbreak of food poisoning arising from a *Salmonella* infection in dried baby milk cost the company concerned £22m. This figure reflects the difference in the value of the company before and after the outbreak (Rowe *et al.*, 1987).

1.3 BACTERIAL PATHOGENS RESPONSIBLE FOR FOOD-BORNE ILLNESSES.

Food-borne illnesses which have arisen from bacterial infection may occur in 2 ways, either the pathogenic bacteria are associated with the food, for example the

association of *Salmonella* with poultry, or the food has become 'cross-contaminated' following contact with contaminated food. Moreover, foodstuffs can be grouped into 'high' and 'low' risk categories. Foods, such as meat or vegetables, that are thoroughly cooked, so that any associated pathogens are killed, should not cause food-borne illness. However, in-effective cooking can result in survival of some pathogens which may subsequently cause illness. Such food can be classified as low risk. Foods that are uncooked, in particular fresh salad vegetables, can carry viable pathogens that are not killed in a cooking step. In addition, cross-contamination of foods that are not thoroughly cooked with foods such as raw meat, result in an increased risk of food-borne illness. Foods that have been previously cooked and stored in inappropriate conditions, such as inadequate refrigeration, also present an increased risk of illness. Such foods are classed as high risk. It should also be noted that the risk of illness from food varies with the particular group of consumer. For example, the risk increases for young children, the elderly and those with compromised immune responses.

Some bacterial pathogens are associated with a wide range of foods, for example *S. typhimurium* has been associated with food-borne illness arising from consumption of raw and cooked meats, vegetables and chocolate (US FDA, 1992). In contrast, *Bacillus cereus* are usually isolated from rice or pasta sources (Gilbert & Taylor, 1976). In general, foodstuffs that have been thoroughly cooked and are served soon after cooking present the lowest risk to the consumer. On the other hand, fresh, uncooked food, or food that may have been in contact with contaminated food, presents a very high risk of associated food-borne illness.

A large number of bacterial pathogens lead to food-borne illness through food contamination. Some well-known bacteria that can cause food-borne illness include *Salmonella*, *Escherichia coli*, and species of *Campylobacter*, *Listeria* and *Bacillus*. *S. enterica* and *Campylobacter* are the most common bacterial pathogens (of humans) isolated from poultry carcasses. The percentage of poultry that contains these pathogens varies according to agricultural practices, hygiene conditions during

slaughter and subsequent handling (Varnam & Evans, 1996). Bacterial pathogens may also be associated with contamination of raw meat. In particular *E. coli* serotype O157:H7 has recently emerged as a serious food-borne pathogen, due to the high potency of the verotoxin it produces. Furthermore, the infective dose of *E. coli* O157 is very low, less than 500 organisms, compared to approximately 10^7 organisms for a pathogen such as *S. typhimurium* (Blaser & Newman, 1982). However, the prior history of the organisms before ingestion can modify the infectious dose by several orders of magnitude. Contamination of dairy products, such as soft cheese often arises as a result of association of *Listeria monocytogenes*. Listeriosis (the disease caused by *Listeria*) primarily affects pregnant women and neonates, although this group is more likely to be susceptible to bacterial pathogens. An important feature of *L. monocytogenes* is its ability to grow at refrigerated temperatures, which allows for selective enrichment for this pathogen (Bayles *et al.*, 1996). Fish and shellfish belong to a group that potentially carry a high risk of infection from pathogens such as *Vibrio cholerae* and *Clostridium botulinum*. In particular, inadequate cooking and consumption of raw fish increase the risk of food-borne illness from this source (Blake *et al.*, 1980; Ball *et al.*, 1979).

1.3.1 Food poisoning arising from associated salmonellae.

Contamination of foodstuffs by salmonellae may arise at any point during the food chain. *S. typhimurium* is mainly associated with raw and cooked meats although it can be present in a broad range of foods. In contrast, *S. enteritidis* is frequently associated with raw eggs, although some of the contamination arises from damaged or broken eggshells. Contamination arising from certain strains results from transovarian infection. In particular, *S. enteritidis* phage types 4, 8 and 13a are able to invade the ovaries and oviducts of chickens, thereby entering the egg prior to shell development (US FDA, 1992).

Animal carcasses that have not been entirely decontaminated provide vehicles for salmonellae to enter the food chain. Undercooked foods and cross-contaminated

foods are a common source of salmonella infections. Indeed, cross-contamination is a common source of bacterial food poisoning in general, from either inappropriate storage or handling where raw meat can come into direct contact with cooked meat products.

It is interesting to note that some of the pathogens that cause gastro-enteritis in humans cause different diseases in animals, or may not cause disease at all in animals. For instance, *S. typhimurium* causes gastro-enteritis in humans but causes a typhoid-like disease in mice (Pang *et al.*, 1995). The O157 serotype of *E. coli* causes serious and often fatal disease in humans but is carried, apparently asymptotically, in healthy adult cattle (Orskov *et al.*, 1987).

1.3.2 *Salmonella*: classification and differentiation.

The genus *Salmonella* is a member of the *Enterobacteriaceae* and is closely related to *Escherichia*. Salmonellae are rod shaped, Gram negative and are motile by means of flagellae. They are responsible for diseases in humans and animals. Members of this genus have been reclassified as belonging to a single species, *Salmonella enterica*, composed of different serovars, for example, *enterica* serovar Typhimurium. To date, a total of 2213 different *Salmonella* serovars have been identified. They are grouped according to their host adaptation, as follows: group 1, e.g. *S. typhi*, only cause disease in humans; group 2, e.g. *S. dublin*, cause disease in some animals and rarely in humans; group 3 contain the remaining strains, e.g. *S. typhimurium* (WHO fact sheet 139, 1997). As with other *Enterobacteriaceae*, such as *Escherichia*, salmonellae are serotyped according to which antigens they present, O is the somatic antigen, Vi the capsular antigen and H the flagellar antigen. In the case of *Salmonella*, differences in the O antigen composition provides the major source of immunological variation. In addition, phage typing is commonly used for many serovars, which differentiates the serovars based on different lipopolysaccaride (LPS) composition. Together, these techniques are used as the primary methods to differentiate salmonellae. Other techniques such as bacteriocin typing, in which the bacteriocins produced by a specific strain are characterised, and genetic typing, which includes plasmid and chromosomal profiling, may be used in association with

conventional techniques. For the purposes of this thesis, the serovars of the subspecies *enterica* will be referred by their serovar designation, for example *S. typhimurium* and *S. enteritidis*.

1.3.3 Infection by *Salmonella*: hosts and symptoms of salmonellosis.

It has been estimated by WHO that *Salmonella* serovars are responsible for 1.3 billion cases of gastro-enteritis and diarrhoea worldwide, annually (WHO fact sheet 139, 1997). Of all the *Salmonella* serovars, *S. typhimurium* is one of the most prevalent agents of food-borne illness in developed countries and this can be seen from statistics for England and Wales (table 1.1). *S. typhimurium* can cause disease in a large range of hosts, including humans, most farm and domestic animals, and birds.

Year	*S. typhimurium*	*S. enteritidis* (PT4)	Other serotypes	Total salmonellas
1981	3992	1087 (395)	5172	10251
1982	6089	1101 (413)	5132	12322
1983	7785	1774 (823)	5596	15155
1984	7264	2071 (1362)	5392	14727
1985	5478	3095 (1771)	4757	13330
1986	7094	4771 (2971)	5111	16976
1987	7660	6858 (4962)	6014	20532
1988	6444	15427 (12522)	5607	27478
1989	7306	15773 (12931)	6919	29998
1990	5451	18840 (16151)	5821	30112
1991	5331	17460 (14693)	4902	27693
1992	5401	20094 (16987)	5860	31355
1993	4778	20254 (17257)	5618	30650
1994	5522	19371 (13782)	7518	30411
1995	6743	16044 (12482)	6527	29314
1996	5542	18256 (13127)	5185	28983
1997	4778	23008 (15266)	4810	32596
1998	2994	16196 (10172)	4230	23420

Table 1.1 The number of cases of salmonellosis arising from different serovars of *Salmonella* in humans. This includes *S. typhimurium*, *S. enteritidis* (the number of cases of *S. enteritidis* caused by phage type 4 (PT4) are shown in parenthesis), and remaining *Salmonella* serotypes. The data was collected for England and Wales from 1981 to 1998. Source: PHLS Laboratory of Enteric Pathogens (1998).

The enteritis caused by Group 3 salmonellae is normally termed salmonellosis. In general, the symptoms develop after an incubation period of 12 to 36 hours, although periods of several days have been reported for the development of *S. typhimurium* infections. Salmonellae are able to replicate both extracellularly, in the gut lumen and the lamina propria, and intarcellularly, in epithelial cells and macrophages. *S. typhimurium* enter the villi of the ileum and multiply in membrane bound vacuoles. Studies with murine intestinal loops and tissue culture have shown that *S. typhimurium* are capable of invading M cells (specialised microfold cells) or epithelial cells by rearrangement of the apical cell membrane, a phenomenon known as membrane ruffling (Wallis *et al.*, 1990). It seems that generally the bacteria do not replicate in these cells, but translocate to adjacent cells. *S. typhimurium* causes a typhoid-like disease in mice, but in general does not normally progress further than the submucosal layer in humans. However, it should be noted that cellular entry in humans may be different.

Invasion of *Salmonella* causes enteritis and fluid secretion (Pang *et al.*, 1995) and the symptoms of human salmonellosis are usually characterised by acute onset of fever, abdominal pain, and diarrhoea, followed by nausea and sometimes vomiting. Fortunately, salmonellosis is usually relatively mild and self-limiting, although in the very young, old and immuno-compromised, dehydration arising from salmonellosis can be life threatening. It is of note that the mortality rate for the elderly is approximately 3% whilst the general figure for the whole population is less than 0.1% (Varnam & Evans, 1996).

1.3.4 *S. typhimurium* DT104 and multidrug resistance.

S. typhimurium also occurs in many animal hosts and therefore poses a significant zoonotic threat. The data in table 1.2 indicates that the incidence of *S. typhimurium* DT104 in farm animals has increased in the UK during 1990s. An important finding is that there is a similar level of incidence in both calves and in adult cattle. Moreover, many animals are asymptomatic carriers of DT104 and may shed

organisms for a number of months. This can result in infection of a healthy population of animals following association with the carrier (Hogue *et al.*, 1997). The recent emergence of the antibiotic resistant strain of *S. typhimurium*, DT104 R-type ACSSuT (resistance pattern to ampicilin, chloramphenicol, streptomycin, sulphonamides and tetracycline) is particularly worrying and reinforces the need to reduce the opportunity of transmission via the food chain. Molecular studies have indicated that the genes for resistance are chromosomally encoded, rather than plasmid based.

Type of animal	Percentage of *S. typhimurium* isolates from farm animals that are DT104 (actual numbers/total numbers of cases)	
	1993	1995
Adult cattle	45 (96/211)	70 (368/528)
Calves	45 (300/663)	72 (970/1347)
Swine	13 (32/252)	36 (101/283)
Sheep	42 (18/43)	70 (86/122)
Poultry	4 (10/220)	52 (174/334)

Table 1.2 *S. typhimurium* isolates from a range of farm animals in the UK. The numbers in parenthesis indicate the actual number of DT014 infected animals and the total number of animals infected with *Salmonella* (Hogue *et al.*, 1997).

1.4 BACTERIAL ADAPTIVE RESPONSES TO SUB-OPTIMAL CONDITIONS

Important food handling targets of many governments include stringent rules aimed at reducing the risks of cross-contamination. Even though correct storage of refrigerated foodstuffs reduces the risk of bacterial growth on the food, bacteria can nevertheless persist at temperatures that are below that permissible for growth. Furthermore, if the temperature is allowed to increase to a level that permits the resumption of bacterial growth, the risk for food-borne illness then increases. Thus, the processes that allow bacteria to adapt to the sub-optimal conditions underpin their ability of cause food poisoning.

The ability of bacteria to adapt to environmental challenges has been studied for many years. In fact, more than a century ago, Professor J. Forster (University of Amsterdam), noted that micro-organisms were able to survive and grow in partly frozen canal water (cited in Mossel *et al.*, 1981). This came as a surprising discovery at the time, since micro-organisms were thought to grow only in their animal hosts, at 'blood temperature'.

Bacteria respond to various environmental stresses in a manner that allows adaptation to the change in environment. Adaptive mechanisms involve transient changes that enable the bacteria to survive in sub-optimal conditions, including changes in protein expression and cell morphology. In some circumstances growth and division can proceed under sub-optimal conditions, after a period of adjustment. These adaptations can also provide cross-protection against other, distinct, stresses. Stress responses to temperature increases and changes in osmolarity are relatively well characterised in Gram negative bacteria. Characterisation of these responses has shown that the role of some proteins is essential to the adaptive process. On encountering a decrease in temperature, it is clear that a stress response is initiated in Gram negative bacteria. This is termed the cold shock response. Although the phenomenon of survival at low temperatures has been investigated for many years, the molecular basis of the cold shock response has only recently been studied. Understanding the cold shock response is particularly relevant to food-borne diseases where pathogens, such as salmonellae, survive in foodstuffs at refrigerated temperatures and subsequently pose a risk to the consumer.

The molecular basis of various stress responses has been well characterised for challenges such as the heat shock response, the osmotic shock response and the oxidative shock response. In addition, it is important to bear in mind that entry into stationary phase is also a stress response since it is preceded by limiting factors, such as starvation. In line with environmental stresses, entry into stationary phase is also characterised by adaptive changes. Generalised pathogens, by their very nature, will encounter a wide range of environments and therefore must adapt to these changes in

order to survive. For example, a gastro-intestinal tract provides very different challenges to seawater. This section reviews some of the responses that are induced by these widely varying environments.

1.4.1 The heat shock response.

The heat shock response is initiated following an increase in the ambient temperature above the optimum temperature for growth. In *E. coli,* approximately 30 genes are involved in the heat shock response. The response is predominantly regulated by the alternative sigma factor, σ^{32}, which is encoded by *rpoH*. Heat shock proteins that are rapidly induced to a high level following the temperature increase, include GroEL, GroES, DnaK and DnaJ. These proteins act as protein chaperones, refolding misfolded proteins or prompting their degradation. The heat shock response is also initiated, at least in part, by other stresses such as ethanol, changes in pH and oxidation (for a review see Bakau, 1993).

1.4.2 The osmotic shock response.

Changes in osmolarity are frequently encountered by generalised food-borne pathogens. Genetic analysis has shown that approximately 20 genes are subject to osmotic control in *Enterobacteriaceae*. Adaptive mechanisms to an increase in osmolarity include uptake of osmoprotectants (also termed compatible solutes), that maintain a constant ionic concentration in the cell. Such compatible solutes are either synthesised *de novo*, or transported into the cell. For example, expression of the *proU* operon is enhanced following an increase in osmolarity. This locus encodes a transport system for the osmoprotectants glycine betaine and proline (for a review see Csonka & Hanson, 1991).

1.4.3 The oxidative shock response.

An increase in the level of oxidation also results in a stress response. For example, following entry of *S. enterica* into macrophages, the bacteria encounter an oxidative burst of highly reactive oxygen species. Normally, such a reaction would be highly toxic to the bacteria. However, initiation of the oxidative response allows adaptation to the hostile environment. Hydrogen peroxide and superoxide initiate two distinct stimulons, which comprise approximately 30 to 40 genes. The key regulators of these responses are OxyR and SoxRS (Morgan *et al.*, 1996)

1.4.4 Growth phase effects.

The growth phase of bacteria has a profound effect on survival when organisms encounter environmental stresses. During periods of stress such as starvation or temperature reduction, non-differentiating bacteria, such as *E. coli* and *S. typhimurium*, that do not form spores, undergo morphological changes and enter into a maintenance state, termed stationary phase (Smith *et al.*, 1994). In this state, the cells exhibit minimal growth, if any. Yet they remain metabolically active and maintain their viability for significant periods of time. If suitable components for growth, such as nutrients, are provided to cells in this state, they may exit from stationary phase and resume growth and division, i.e. they may re-enter exponential phase. There is growing evidence that stationary phase cultures become highly resistant to environmental stress (Kolter *et al.*, 1993). On entry into stationary phase *E. coli* cells are observed to lose their rod shape, becoming almost spherical, as the result of several cell divisions without an increase in cell mass. In comparison, in stationary phase, marine bacteria become greatly reduced in size by a combination of reductive divisions and endogenous metabolism, and are termed ultra-microcells. Cell membranes change becoming less fluid and less permeable due to fatty acid composition changes. The nucleoid condenses and specific patterns of proteins are expressed depending on the conditions that induced entry into stationary phase (Kolter *et al.*, 1993). In *E. coli* a core set of 15-30 proteins, known as the Pex (post-

exponential) proteins, is always induced, in addition to those expressed specifically in responses to conditions encountered (Kolter *et al.*, 1993).

The alternative sigma subunit of RNA polymerase, σ^s, encoded by *rpoS* and expressed during entry into stationary phase, is a global regulator which is necessary for both positive and negative regulation of some of the Pex proteins (Lange & Hengge-Aronis, 1991). In addition to global regulation by σ^s, there are other regulatory factors involved in stationary phase expression. cAMP stimulates the induction of several starvation induced genes (Loewen & Hengge-Aronis, 1994) on one hand, and negatively regulates some *pex* genes and several σ^s dependent genes on the other hand (Land *et al.*, 1993). σ^s is now known as a general stress σ factor and is induced under conditions other than stationary phase (Hengge-Aronis 1996). A recent review listed as many as 50 genes regulated by σ^s (Loewen *et al.*, 1998) and a number of these are shown in table 1.3. *S. typhimurium rpoS* appears to be homologous to *E. coli rpoS*. However, additional, unique roles for the former have been identified, for example regulation of the plasmid based *spv* operon, which is involved in virulence (Kowarz *et al.*, 1994). Table 1.3 lists some of the σ^s regulated proteins of *E. coli* and *S. typhimurium* and their associated functions (Loewen & Hengge-Aronis, 1994, Loewen *et al.*, 1998).

σ^s positively regulated genes	Function
katE, katG, xthA, dps, aidB	prevention and repair of DNA damage
bolA, ficA	cell morphology
spvABCD, spvR, csgA	modulation of virulence in *Salmonella, Shigella* and *E. coli*
otsBA, treA, csiD, htrE	osmoprotection and thermotolerence
glsS	glycogen synthesis
appY, appCBA, hyaABCDEF	involved in anaerobic respiration
osmB, osmY, cfa	involved in membrane and cell envelope functions

Table 1.3 A selection of some of the σ^s regulated genes in stationary phase, and their associated functions.

1.4.5 The role of other regulators in late exponential phase gene expression.

When cells enter stationary phase gene expression is regulated in a complex, cascade manner. Regulation by the stationary phase sigma factor has been described and in addition, global regulators, such as CRP (cyclic AMP receptor protein), IHF (integration host factor) and Lrp (leucine response regulator protein) play important roles in the regulation of expression of stationary phase genes. When Lange and Hengge-Aronis (1991) identified σ^s, they found that it is negatively controlled by cAMP. *E. coli* contains more than 100 different promoters that can be activated by cAMP (Savery *et al.*, 1996), in most cases the CRP acts by making direct contact with RNA polymerase. IHF is a histone like protein that is induced on entry into stationary phase, independently of *rpoS* and Lrp is a central positive regulator for gene expression after a nutritional downshift (Landini *et al.*, 1996). Lange *et al.* (1993) found that *osmY*, which is strongly induced in a σ^s dependent manner on entry into stationary phase, is also under the control of global regulators Lrp, IHF and cAMP-CRP, (*osmY* encodes a periplasmic protein of unknown function). Lrp was found to repress expression of *osmY* on entry into stationary phase, in rich media. cAMP was found to greatly repress *osmY* expression during late exponential phase, when the growth rate begins to slow down, however, this was not the determining factor in the final level of *osmY* expression in stationary phase (Lange *et al.*, 1993). This study illustrates the complex levels of gene expression regulation on entry into stationary phase.

1.5 SURVIVAL AND GROWTH OF MICRO-ORGANISMS AT LOW TEMPERATURES

The ability of micro-organisms to persist in refrigerated conditions underpins their potential to cause food poisoning and food spoilage. Persistence at low temperatures requires adaptations that allows survival. These adaptive mechanisms of the micro-organisms are investigated in relation to conditions that allow optimal growth, i.e. laboratory conditions. The growth rate of bacteria is governed by several factors,

including temperature. In general, bacteria can grow over a temperature range of 30°C or 40°C. Bacteria are classified according to the specific temperature range over which they can grow: thermophiles grow at elevated temperatures above 55°C and include *Bacillus stearothermophilus*, mesophiles grow optimally within the temperature range 20°C – 45°C, and include *S. typhimurium* and *E. coli*, psychrophiles such as *Bacillus cereus* grow within a temperature range of 20°C to less than 0°C (Stanier *et al.*, 1984) (It should be noted that the temperature ranges used in this system of classification are approximate). The temperature ranges over which bacteria are able to survive, but are unable to grow and divide, are far greater than the growth ranges. Temperatures outside the optimal range induce stress responses, termed the heat shock response and the cold shock response. These responses are induced at different temperatures which is largely dependent on which class the bacteria belong to.

1.5.1 Factors that determine temperature limits for growth.

Under optimal conditions, including optimal temperature, nutrient supply, the appropriate level of aeration and osmolarity, bacterial growth proceeds logarithmically as predicted by the Arrhenius equation (Arrhenius, 1889).

$$r = A \exp. - ^{(Ea/RT)}$$

The equation describes the exponential dependence of the rate constant on temperature for a large number of chemical reactions. In the equation, r represents the bacterial growth rate, A represents the Arrhenius constant, exp represents the exponential, Ea represents the activation energy (sometimes also known as μ), T represents the absolute temperature and R is the universal gas constant.

Figure 1.1 shows a typical Arrhenius plot for a mesophile, such as *S. typhimurium*, which grows optimally between 25°C and 40°C. The plot is only linear over a portion of the temperature range for growth. It can also be seen that growth rate falls abruptly at both the upper and lower limits of the temperature range. Adaptive responses are associated with such changes in growth rate, termed the heat shock and cold shock

response, respectively. Consequently, although bacteria may have a limited range of temperature at which optimal growth occurs, sub-optimal growth occurs over a far greater temperature range. The minimum growth temperature for *S. typhimurium* is reported to be approximately 7°C (Mossel *et al.*, 1981). However, differences of 1°C or 2°C occur in the minimum growth temperature of different strains. This may be influenced by growth conditions.

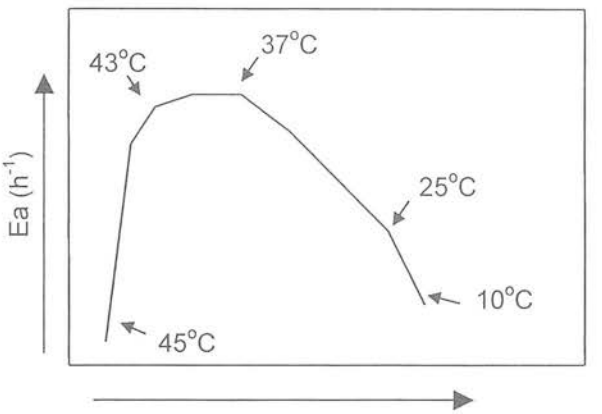


Figure 1.1 Arrhenius plot of growth rates of a typical mesophile, such as *S. typhimurium*. The log of the specific growth rate constant Ea (per hour) was plotted against the reciprocal of absolute temperature (K) (Stanier *et al.*, 1984).

The maximum temperature for growth is governed by protein misfolding, thermal denaturation of proteins and possibly by loss of function of cell structures such as membranes, ribosomes and polymerases. Growth cannot proceed when the temperature exceeds a point at which these destructive reactions become overwhelming. At low temperatures, proteins undergo a slight conformational change. This is due to the weakening of hydrophobic bonds that play a fundamental role in determining tertiary structure. The conformational changes which occur in proteins and nucleic acids, together with changes in membrane fluidity, determine the minimum growth temperature of bacteria (Stanier *et al.*, 1984).

1.5.2 Changes in membrane fluidity.

Growth and survival at low temperatures present several challenges for bacteria to overcome. Following temperature variations, changes in membrane fluidity occur that must be overcome in order that membrane function is maintained or restored.

Exposure to a rapid temperature downshift leads to a reduction in membrane fluidity, whereas membrane fluidity increases following exposure to heat shock. During adaptation to low temperature, the proportion of *cis*-unsatuarated fatty-acyl groups in the membrane lipids increases and this is brought about by induction of fatty acid desaturase activities (Vigh *et al.*, 1998). This, in turn, leads to an increase in membrane fluidity and restoration of membrane function (Panoff *et al.*, 1998). The mRNA of *desA*, which encodes a fatty acid desaturase in the cyanobacterium *Synechocystis*, was shown to increase dramatically following a shift from 37°C to 22°C (Vigh *et al.*, 1998). In addition, disruption of the *des* gene of *B. subtilis* resulted in the failure to synthesise unsaturated fatty acids following a temperature reduction from 37°C to 20°C. Moreover, survival of the *des* mutant strain was reduced, relative to the wild type strain, when the culture was in stationary phase (Aguilar *et al.*, 1998).

1.5.3 Changes in ribosome capacity.

In addition to changes in membrane fluidity, protein translation initiation is blocked following a temperature reduction. When a culture of *E. coli* was decreased from 37°C to 5°C, crude cell free extracts incorporated amino acids into protein for approximately 30 minutes, and at a rate of approximately 12 % that seen at 25°C. Polysomal runoff occurred within 60 minutes of the temperature reduction, followed by accumulation of 70S ribosomal subunits. Further *in vitro* studies showed that some of the 70S particles, which accumulated at 5°C, were 70S ribosomes which contained mRNA either attached or in association. Furthermore, the ribosomes were shown to be capable of polypeptide elongation at 25°C (Broeze *et al.*, 1978).

1.5.4 Changes in DNA supercoiling.

Changes in supercoiling have been proposed to influence gene expression during the cold shock response. It has been shown that the negative supercoiling of plasmid DNA (pUC18) in *E. coli* cells increased following a shift from 37°C to 6°C. In

addition, the increase in negative supercoiling was found to be transient, so that the plasmid DNA reverted to its original state within 60 minutes of the temperature shift. The recovery of the physical state of the DNA was termed the re-relaxation reaction (Mizushima *et al.*, 1997).

1.5.5 Global changes in protein expression.

Many studies, with different species of bacteria, have shown a similar pattern of change in the synthesis of proteins following temperature reduction. In general, if the temperature is shifted to a point close to, or just above the minimum temperature permissible for growth, overall protein synthesis initially decreases, before returning to levels that are similar to those observed prior to the shift. In many bacteria, during the initial phase when protein translation declines, one or more proteins are expressed to very high levels. The increase in expression is frequently higher than expression at non-stress temperatures. Following adaptation, the expression of these cold induced proteins returns to a lower, basal level. Together, these changes reflect the key process of adaptation to growth and/or survival at low temperatures, known as the cold shock response.

1.6 THE COLD SHOCK RESPONSE

Studies of the cold shock response have been carried out in many species of bacteria and in *E. coli* and *B. subtilis* in particular. (The majority of studies have monitored the response of the bacteria when in exponential phase of growth). The key features of adaptation to low temperature apply to most of the bacteria. In addition, in cultures of some bacteria, including *E. coli*, a lag in growth has been observed immediately following the shift to the low temperature (Jones *et al.*, 1987). In contrast, no growth lag has been observed in *B. subtilis*, a variety of strains of *Lactobacillus lactis* and *Enterococcus faecalis* (Graumann *et al.*, 1996, Kim & Dunn, 1997, Panoff *et al.*, 1997).

Studies have shown that chilling cultures of *Bacillus subtilis* at 10°C for 2 hours increased the survival of cells (measured in terms of plating viability) that were subsequently frozen at -80°C. Survival of cultures that were incubated at 10°C prior to freezing was approximately 97 %, an increase from approximately 25 % for the cultures that had not been chilled prior to freezing. Moreover, deletion of the major cold induced protein of *B. subtilis*, CspB, resulted in a further decline in plating viability. Survival of cells of the *cspB* mutation that had been incubated at 10°C prior to freezing at –80°C decreased to approximately 44 %. In contrast, survival of the culture that had not been pre-adapted prior to the freezing, decreased to approximately 3 % (Willimsky *et al.*, 1992). These studies provide evidence that the cold shock response plays an important adaptive role and that CspB plays a key role in this process.

Following a temperature downshift from 37°C to 15°C of a culture of exponentially growing *E. coli* cells, Jones *et al.* (1987) found that selective induction of a set of cold shock inducible proteins occurred. During the initial 2 hours, overall protein synthesis was greatly depressed whilst at the same time a set of proteins was specifically induced. This period is sometimes called the acclimation phase. Included in this initial set of cold shock proteins, a novel protein was very highly induced which was barely detectable prior to the temperature reduction. This protein is the major cold shock protein, termed CspA (cold shock protein A). The majority of the cold inducible proteins were induced between 2- and 10- fold, in comparison to the 100-fold induction of CspA. Proteins that were transiently induced during this period are sometimes termed CIPs (cold induced proteins). Jones & colleagues (1987) also found that after 4 hours at 15°C, the overall pattern of protein synthesis had recovered to a level close to that prior to the downshift and that the level of CspA had reduced to a new, basal level. In addition, after the 4 hour incubation synthesis of some proteins remained at higher levels than prior to the temperature shift. Such proteins were termed CAPs (cold acclimation proteins).

Further studies on *E. coli* proteins induced following temperature downshifts from 37°C to 15°C and 37°C to 10° C have resulted in the identification of additional cold inducible proteins. Table 1.4 lists the *E. coli* proteins that are known to be induced following a temperature downshift. In addition, several induced proteins remain unidentified. Cold induced proteins are involved in a wide range of cellular processes. This includes several proteins that are involved in DNA supercoiling. This is in line with the observations by Mizushima *et al.*(1997), that negative supercoiling of plasmid DNA transiently increases following a temperature shift from 37°C to 6°C. *E. coli* DNA gyrase is a type II topoisomerase that catalyses the negative supercoiling of DNA when coupled to ATP hydrolysis (Wang, 1985). The GyrA subunit is induced following a temperature reduction and assuming that superhelicity of the nucleoid is significant, the lag time of 4 hours before growth resumes when *E. coli* is shifted to 10°C is consistent with the time taken for gyrase synthesis to be increased after cold shock (Jones *et al.*, 1992). In other words, the synthesis of GyrA increases during the lag period. Thus changes in DNA supercoiling occur which may subsequently play a role in the resumption of overall protein synthesis during the acclimation period.

Table 1.4 Known *E. coli* cold inducible proteins and their functions

General Function	Protein	Protein Function
DNA STRUCTURE	GryA[1]	Introduces negative supercoils into DNA helix
	H-NS[1]	Involved in chromosome condensation and transcriptional silencing
RECOMBINATION	RecA[1]	Involved in recombination and the SOS response
TRANSCRIPTION	NusA[1]	Transcriptional anti-terminator
TRANSLATION	CsdA[3]	Contains a DEAD box motif, RNA helicase.
	IF2 - α[1]	IF2 binds to charged tRNA-f-met to 30S ribosomal subunit
	IF2 - β[1]	
	Pnp[1]	Degradation of mRNA
	RplL[2]	Ribosomal protein

Table 1.4 continued

	RbfA[4]	Involved in ribosomal maturation or initiation of translation
PROTEIN FOLDING	Hsc 66[6]	Heat shock protein (DnaK) homologue
	IbpB[2]	HSP20 homologue
	Trigger factor[5]	Chaperone associated with GroEL
METABOLISM	AceF[1]	Decarboxylation of pyruvate
	AceE[1]	Dihydrolipoamide acetyltransferase subunit of pyruvate dehydrogenase
	SucA[2]	Components of 2-oxoglutarate dehydrogenase
	SucB[2]	Component of succinate dehydrogenase
	SdhA[2]	
	CarA[2]	carbamoyl-phosphate synthetase
	MetE[2]	tetrahydropteroyltriglutamate methyltransferase
	ArgI[2]	ornithine carbamoyltransferase I
MEMBRANE FUNCTION	OmpA[2]	Outer membrane protein
STRESS RESPONSES	SodA[2]	Superoxide dismutase
	CspA[7]	Major cold shock protein
	CspB[8]	CspA homologue
	CspG[9]	CspA homologue
	CspI[10]	CspA homologue

References - Jones *et al.*, 1987[1], Herendeen *et al.*, 1979[2], Jones *et al.*, 1996[3], Jones & Inouye, 1996[4], Kandror & Goldberg, 1997[5], Leilivelt & Kawula, 1995[6], Goldstein *et al.*, 1990[7], Lee *et al.*, 1994[8], Nakashima *et al.*, 1996[9], Wang *et al.*, 1999[10].

Some of the cold induced proteins are involved in transcription and translation. It is plausible that these proteins are translated during the acclimation phase so that transcription and translation can proceed once the cell has adapted to the low temperature. The same is possibly true for the synthesis of proteins that are involved

in metabolism. Finally, it is of note that several homologues of the major cold shock protein have been found to increase in *E. coli* cells during cold shock. The history of their identification, possible roles and regulation will be discussed.

1.6.1 The CspA family – a novel group of highly homologous proteins.

CspA is the major cold shock protein of *E. coli* and belongs to a family of nine highly related proteins, termed the CspA family. Cold shock inducible members of the CspA family are sometimes termed CSPs (cold shock proteins), although they will be referred to as CspA homologues in this thesis. Following identification of *E. coli* CspA, many homologues have been identified in other bacteria, including 4 homologues in *S. typhimurium*. The proteins are generally around 7.5 KDa in size, contain around 70 amino acids and in the majority of cases share > 70 % amino acid sequence identity. Members of the *E. coli* CspA family generally share > 65 % amino acid sequence identity to CspA, however, CspD is the least homologous protein and shares 45 % sequence identity to CspA. Table 1.5 lists bacteria that are known to contain more than one member of the CspA family. Cold shock inducible members of the CspA family have been highlighted, where known. CspA-like proteins are very highly conserved and have been identified in most bacteria, either through database searches of genomes or using molecular detection methods. Nevertheless, some organisms appear not to contain any homologues. The known exceptions, to date, are *Campylobacter jejuni* (this sequence is not yet complete and the sequence data were produced by the *C. jejuni* NCTC 11168 Sequencing Group at the Sanger Centre and can be obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/cj/CJ.pep), *Helicobacter pylori* (Tomb *et al.*, 1997) and *Mycoplasma* (Fraser *et al.*, 1996). It is interesting to note that *Mycoplasma* bacteria are highly host adapted and therefore, may not encounter temperature extremes to the same extent as those bacteria that possess CspA homologues.

BACTERIA	CspA HOMOLOGUES
Salmonella typhimurium	CspA, CspB, CspG, CspH
Escherichia coli	CspA, CspB, CspC, CspD, CspE, CspF, CspG, CspH, CspI
Bacillus subtilis	CspB, CspC, CspD
Bacillus cereus	CspA (U60028), CspB (X93040), CspC (X93041), CspD (X93042), CspE (X93043)
Clostridium acetobutylicum	CspA (Z37723), CspB (Z50008), CspC (Z50033), CspD (Z50009)
Lactococcus lactis	CspA (Y17215), CspB (Y17215), CspC (Y17216), CspD (Y17216), CspE (Y17217)
Listeria monocytogenes	CspL (X91789), CspLB (U90213)
Staphylococcus aureus	CspA (U60050), CspB (Af003592), CspC (Af003593)
Strepococcus agalactiae	CspA (L09116), CspB (L09116), CspC (L09116), CspD (L09116)
Yersinia entercolitica	CspA1 (U82821), CspA2 (U82821)

Table 1.5 Bacteria that contain at least 2 different CspA-like proteins. Those proteins that are under-lined are known to be cold shock inducible. The accession numbers of the proteins are given in parenthesis after the protein name. This search was performed using the GenEMBL database, April 1999.

1.6.1.i Identification and characterisation of members of the *S. typhimurium* CspA family.

S. typhimurium CspB was identified in our laboratory, following detection of low temperature inducible Mu*dlux* fusion isolates. The Mu*dlux* element was randomly inserted into *S. typhimurium* and the pool was screened for bioluminescence following temperature reduction from 37°C to 10°C. Further light reporter studies and RNA analysis indicated that CspB induction was subject to a 'temperature switch' at approximately 22°C, and that the level of *cspB* mRNA increased as the temperature was reduced further (Craig *et al.*, 1998). *S. typhimurium* CspA was detected from a Mu*d*P22 lysate of *S. typhimurium* LT2 DNA using a probe corresponding to the open reading frame (ORF) of *E. coli cspA*. The *cspA* gene was cloned and sequenced, and the ORF was shown to share 100 % identity to the *E. coli*

cspA coding sequence (Craig & Gallagher, unpublished data). The amino acid sequence identity of *S. typhimurium* CspB to *S. typhimurium* CspA is 63 %. In addition, EMBL database searches show that CspC (accession number Af052580) and CspH (accession number Af006035) have been detected in *S. typhimurium*. They share 62 % and 47 % amino acid identity to *S. typhimurium* CspA, respectively. The major cold shock protein CspA has been detected in *S. enteritidis* and the *cspA* ORF has been shown to share 100 % identity with the corresponding nucleic acid sequence of *E. coli cspA* (Jeffreys *et al.*, 1998) (table 1.6 and figure 1.2).

CspA homologue	**Amino acid identity to *S. typhimurium* CspA**
E. coli CspA	100
E. coli CspB	79
E. coli CspG	73
E. coli CspI	69
S. typhimurium CspB	63
S. typhimurium CspC	62
S. typhimurium CspH	47

Table 1.6 Amino acid identity of *S. typhimurium* CspA homologues to *E. coli* cold inducible CspA homologues. (These identities were calculated using the alignment tool in the ExPASy Molecular Biology Server website, from Corpet, 1988).

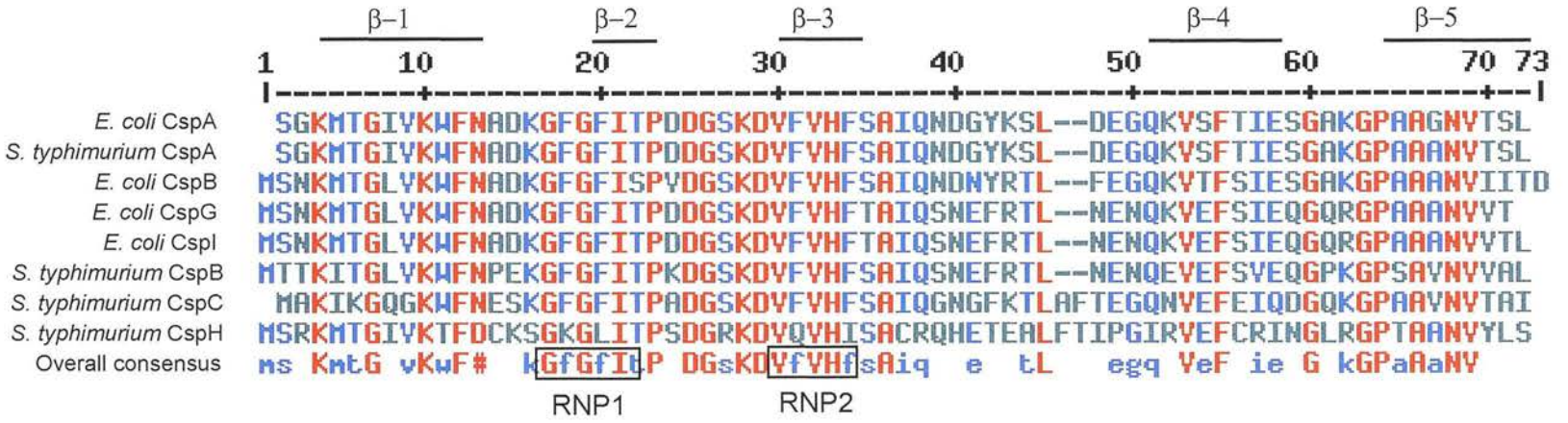


Figure 1.2 Amino acid sequence alignment of *S. typhimurium* CspA, CspB and CspH with cold shock inducible *E. coli* CspA homologues, CspA, CspB, CspG and CspI.

The alignment and the consensus sequence were derived according to Corpet, (1988). Amino acid residues that have high consensus levels (> 90 %) are highlighted in red, while those with low consensus levels (> 50%) are highlighted in blue. The # symbol is any residue of N, D, Q or E. Spaces have been inserted into some of the sequences to optomise the alignment of the protein sequences. The proposed RNP motifs, 1 and 2, have been boxed and the amino acid residues that form beta strands, as elucidated from *E. coli* (Newkirk *et al.*, 1994), have been indicated.

1.6.1.ii Identification and characterisation of members of the *E. coli* CspA family

The nucleic acid sequence of *E. coli cspA* was determined by Goldstein & colleagues (1990). Initially, the CspA protein was isolated from cold-shocked cell extracts (taken when the culture was exponentially growing) and resolved using 2-D PAGE (2-dimensional polyacrylamide gel electrophoresis). Part of the protein sequence was determined by N-terminal sequencing, and from this, an appropriate DNA probe was designed. An *E. coli* plasmid library was probed with the DNA, which resulted in identification of 'positive' clones. The sequence of the *cspA* gene was subsequently determined. The promoter region of *cspA* was identified by deletion analysis and revealed an unusually long 5'-untranslated region (UTR) prior to the open reading frame (Tanabe *et al.*, 1992). In further studies, examination of the Kohara λ phage miniset with a DNA probe corresponding to the *cspA* ORF revealed the presence of 2 *cspA* homologous sequences, termed *cspB* and *cspC* (Lee *et al.*, 1994). In addition, a region upstream of the *clpA* gene was found to encode a small molecular weight protein, with a similar amino acid sequence to CspA, termed CspD. Reporter studies where LacZ was fused to the 10^{th} amino acid codon of *cspA*, *cspB*, *cspC* or *cspD*, showed that both CspA and CspB were induced to similar levels following incubation of exponentially growing cultures of *E. coli*. In contrast, no β-galactosidase activity was detected from either of the translational fusions, *cspC-lacZ* or *cspD-lacZ* (Lee *et al.*, 1994). After incubation for 4 hours at 15°C, the level of induction from *cspA-lacZ* and *cspB-lacZ* reached approximately 16-fold and 18-fold higher than the level observed at 37°C, respectively (Lee *et al.*, 1994). Further studies have shown that *E. coli* CspD is growth phase dependent and reporter studies where *lacZ* was fused to the part of the ORF of *cspD* showed that *cspD* was induced on entry in stationary phase. In addition, studies with a *rpoS* mutant showed that the CspD induction was σ^S –independent (Yamanaka & Inouye, 1997).

E. coli cspE was identified as a multicopy suppressor of a *mukB* mutation. MukB is thought to be involved in chromosomal partitioning and *mukB* mutants produce

normal sized, anucleate cells at a significant frequency (Yamanaka *et al.*, 1994). Later *in vitro* studies on *E. coli* RNA/protein complexes, using a photocrosslinking method that covalently bonds RNA to the proteins it contacts, suggested that the role of CspE in chromosome partitioning may be due to involvement in transcription (Hanna *et al.*, 1998). CspE was shown to interact directly with nascent RNA in transcription complexes from several promoters, including *rrnB* P_2, *rrnG* P_2, λ P_L, λ P_R and λ $P_{R'}$. Purified CspE was also shown to inhibit transcriptional antitermination mediated by the λ Q protein, by approximately 50 %. Furthermore, gel shift assays showed that purified CspE bound to ssDNA (the promoter region of the Q protein) that contained the core Y box sequence ATTGG (see Wolffe, 1993, for a review on Y-box proteins). Reporter studies, monitoring the β-galactosidase activity from a *cspE-lacZ* translational fusion (where *lacZ* was fused to the 13th codon of *cspE*), showed that CspE was continuously expressed at high levels at 37°C, and expression transiently increased (by approximately 50 %) during lag phase, immediately following dilution of the stationary phase culture into fresh medium. Similar results were observed at 30°C and 42°C. Thus, *cspE* appears to be constitutively expressed at optimal temperatures (Bae *et al.*, 1999).

Evidence for expression of CspG at low temperature arose from a separate study (Nakashima *et al.*, 1996). Transformants, carrying random chromosomal DNA fragments inserted upstream of the promoterless *ampC* gene on a plasmid, were screened in the presence of ampicilin, at 15°C. Analysis of the transformants that grew under these conditions revealed the presence of a novel, cold inducible member of the CspA family, termed CspG. Reporter assays, where *lacZ* was translationaly fused to the *cspG* gene at the start of the ORF, revealed that CspG was most highly induced at 15°C.

CspF, CspH and CspI were identified following database searches using the Blast algorithm (Altschul *et al.*, 1990). CspH was also later identified as a homologue of CspF by DNA sequencing of part of the chromosome by Oshima *et al.*, (1996). Further studies using transcriptional and translation fusions and analysis by 2D

PAGE has shown that CspI is also cold shock inducible. For reporter studies, transcriptional and translational fusions of *cspI* were constructed using *lacZ*, which corresponded to either the 5'-UTR only or together with the coding region up to the 13th codon of *cspI*, respectively. Following a temperature shift from 37°C to 15°C, the level of β-galactosidase activity from the transcriptional *cspI-lacZ* fusion was found to be slightly more than that observed for *cspA-lacZ*, while β-galactosidase activity from the translational *cspI-lacZ* was significantly higher than that observed for *cspA-lacZ*. 2D PAGE analysis showed that CspI migrates to a very similar position to that of CspB. Similarly CspG migrates to a comparable position to that of CspA (Wang *et al.*, 1999).

Further studies with *E. coli* have shown that in the presence of 0.2 mg ml^{-1} chloramphenicol, whilst no overall protein synthesis is detected at 37°C, production of CspA, CspB and CspG was evident following incubation at 15°C, for 30 minutes. The effect on overall protein synthesis after incubation at 15°C for 30 minutes in the presence of 0.1 mg ml^{-1} kanamycin was similar to that observed for chloramphenicol, although the production of either CspA or CspG was slightly less (since CspA and CspG migrate to very similar positions, it is difficult to determine whether the production of either one or both had been reduced). RNA analysis in the presence of these antibiotics at 37°C and 15°C indicated that mRNAs for *cspA* and *cspB* were transcribed at the same level as in the absence of the antibiotics (Etchegaray & Inouye, 1999).

1.6.1.iii Identification and characterisation of members of the CspA family in other bacteria.

In general, identification of CspA-like proteins has arisen through analysis of changes in proteome, or through characterisation of DNA that has bound to a generic *cspA*-like DNA probe, or through searches of bacterial genomes for CspA homologues. In some cases homologues have also been detected through cross-reaction to CspA antibodies or through the use of degenerate PCR primers based on the *cspA* gene of

E. coli. The following evidence presented highlights some of the studies that have lead to positive identification of CspA-like proteins. However, as information on bacterial genomes is updated frequently, the data presented represents a sample of the work on cold shock proteins at the time of writing.

Shortly after the initial reports of *E. coli* CspA, 2D PAGE analysis of *B. subtilis* indicated the presence of a CspA-like protein that was induced following a temperature decrease from 37°C to 10°C. The protein, termed CspB, was isolated using 2D gels and the N-terminus was sequenced. It was found to have 61 % amino acid sequence identity to *E. coli* CspA. Reporter studies, where *lacZ* was fused immediately downstream of the RBS (ribosome binding site) of *cspB*, showed that CspB was induced approximately 6 to 8-fold, following a shift from 37°C to 10°C. CspB induction peaked on 2 sequential occasions, the 1st peak occurred after 1 hour at 10°C and the 2nd occurred 2 hours after the shift (Willimsky *et al.*, 1992). Further 2D PAGE analysis and protein microsequencing revealed the presence of 2 additional homologues in *B. subtilis*, CspC and CspD (Graumann *et al.*, 1996). Using the *B. subtilis cspB* DNA as a probe, one *cspB* homologue was identified in each of the thermophilic bacilli *B. caldolyticus* and *B. stearothermophilus*. The CspB proteins from these organisms share 82 % and 84 % sequence identity to *B. subtilis* CspB, respectively (Schroder *et al.*, 1993).

Similar analysis, by 2D PAGE, of the pyscrotrophic bacillus, *B. cereus*, at 7°C and 30°C revealed the presence of a CspA homologue. The protein was sequenced and termed CspA. It exhibited 63 % amino acid identity to *E. coli* CspA. Moreover, antibodies raised against CspA bound to four additional proteins. The proteins were isolated, cloned and sequenced following PCR amplification with primers that corresponded to regions of CspA. These proteins were termed CspB, CspC, CspD and CspE and they each share > 67 % amino acid sequence identity with each other (Mayr *et al.*, 1996).

A 7.0 KDa protein, which shares 56 % amino acid sequence identity to *E. coli* CspA was identified in *Streptomyces clavuilgerus*. The protein, termed SC 7.0, was identified during a study of the threodoxin system of *S. clavuilgerus*, and was found to co-purify with threodoxin. Protein SC 7.0 was N-terminally sequenced, and based on this a mixed degenerate oligodeoxynucleotide probe was constructed. An ORF of 66 codons was detected that bound to the DNA probe (Av-Gay *et al.*, 1992).

2D PAGE analysis indicated the presence of a cold shock inducible CspA-like protein in the psychroptrophic bacterium, *Arthrobacter globiformis*. Subsequently, DNA corresponding to the *cspA*-like gene was detected by primers that were based on the conserved regions of *E. coli cspA* and *B. subtilis cspB*. An antibody raised against a synthetic peptide which corresponded to part of the *cspA*-like sequence was bound by several proteins, although only one of these proteins was characteristic of the CspA family (Berger *et al.*, 1996).

A similar strategy of designing primers based on conserved regions of *E. coli cspA* was used to detect a *cspA* homologue in each of a range of Lactic Acid bacteria. This group of bacteria comprises of *Lactobacillus helveticus, Streptococcus thermophilus, Pediococcus pentosaceus* and several strains of *Lactococcus lactis* (Kim & Dunn, 1997). The deduced amino acid sequence of the *cspA* gene from one particular strain, *L. lactis* M474, was shown to share 68 % identity with *E. coli* CspA (Kim & Dunn, 1997).

As yet, CspA homologues have not been conclusively identified for *Enterococcus faecalis*. However, a partial coding sequence for a *cspA*-like protein is present in the EMBL database. In addition, examination of protein expression by 2D PAGE, following a shift from 37°C to 8°C, demonstrated that the relative intensity of 3 proteins less than 10 KDa increased by 37 to 48-fold, after a 4 day incubation at 8°C. It appears quite likely that at least one of these proteins is a cold shock inducible member of the CspA family (Panoff *et al.*, 1997).

A *cspA* gene in *S. enteritidis* was identified by hybridisation to a DNA probe that corresponded to *E. coli cspA* DNA and presence of the protein was confirmed by hybridisation to an *E. coli* CspA antibody. Protein expression following a downshift in temperature from 37°C to 10°C or 5°C, coupled with 1D gel electrophoresis showed a high level of induction of a protein (or proteins) that was characteristic of a CspA homologue. Antibodies raised against *E. coli* CspA bound to the highly induced protein(s), indicating that at least one CspA homologue was induced 30 minutes after a shift from 37°C to either 10°C or 5°C (Jeffereys *et al.*, 1998).

In a study to detect and identify bacteria using PCR techniques, Francis and Stewart (1997) used degenerate primers that corresponded to a highly conserved region of the *cspA* homologues. With these primers, *cspA* homologues were successfully amplified from more than 30 diverse Gram positive and Gram negative bacteria (Francis & Stewart, 1997).

Genomic database searches have also indicated that many other bacteria possess at least 1 CspA homologue, for example, searches of the *Listeria monocytogenes* genome revealed 2 CspA homologues, CspL (accession number X91789) and CspLB (accession number U90213).

1.6.2 The structure of CspA-like proteins.

The structure of both *E. coli* CspA and *B. subtilis* CspB has been elucidated using X-ray crystallography and NMR (nuclear magnetic resonance) (Newkirk *et al.*, 1994, Schindelin *et al.*, 1994). The proteins are mainly hydrophobic with several aromatic residues localising on the surface of the protein. They form tightly wound 5-stranded β-barrels with intervening loop regions. The main variations between the homologues lie in the variable loop regions. The overall structure is very similar to a domain in staphylococcal nuclease (Hynes *et al.*, 1991). Figure 1.3 shows the elucidated structure of *E. coli* CspA, adapted from Feng *et al.*, (1998). Members of the CspA family share very high amino acid sequence identity, approximately 43 %,

with the central nucleic acid binding domain of the eukaryotic Y box proteins, termed the cold shock domain (Lee *et al.*, 1994). CspA homologues contain RNA binding sites, complete RNP1 (ribonucleo binding protein) and partial RNP2, respectively, which are found on β sheets 2 and 3.

CspA proteins are members of the RNP motif family. The RNP consensus motif, composed of 2 short sequences, RNP1 and RNP2, is the identifying feature of the RNP motif proteins. Examples of these proteins are present in animal, plant, fungal and bacterial cells. Moreover, the proteins are found in nearly all organelles where RNA is present which suggests an ancient protein structure with important functions. The consensus sequences are as follows; RNP1 – KGFGFVXF and RNP2 – LFVGNL. The three-dimensional structures of the RNA binding domain (RBD) of 2 RNP motif proteins, human U1 snRNP A and human hnRNP, have been determined. The secondary structure of the proteins is: βαββαβ and the RNP consensus motif sequences, RNP1 and RNP2, are found on the 3rd and 1st β strands, respectively (for a review see Burd & Dreyfuss, 1994). In a study of 30 diverse Gram negative and Gram positive bacteria, analysis of amino acid sequences of the CspA homologous (deduced from the respective nucleic acid sequences), revealed that the most highly conserved regions of these proteins corresponded to the RNA binding motifs, RNP1 and RNP2 (Francis & Stewart, 1997).

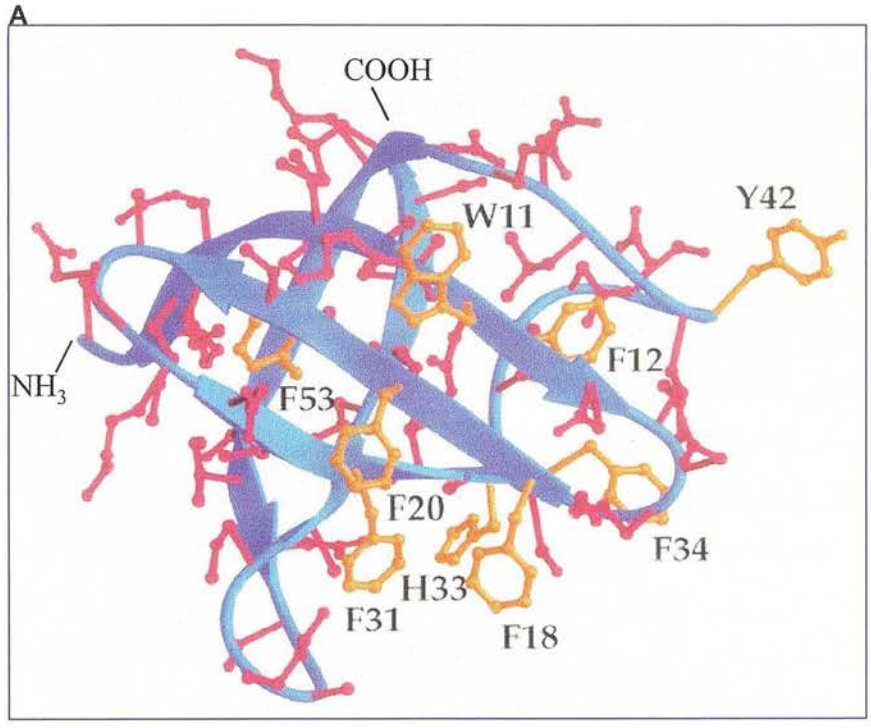


Figure 1.3 Three-dimensional structure of CspA.

A. Solution NMR structure of CspA, adapted from Feng *et al.*, (1998). Aromatic residues localising on the surface are shown in yellow and numbered.
B. Primary amino acid sequence of CspA. The regions corresponding to β 1, β 2, β 3, β 4, and β 5 strands are indicated by lines on the top of the sequence. RNA binding motifs, RNP1 and RNP2, are boxed in red (Newkirk *et al.*, 1994).

Eukaryotic Y-box proteins were initially characterised through their interaction with duplex DNA containing the sequence ATTGG. Further studies on human YB1 showed that the regulatory sequence was CTG<u>ATTGG</u>$^{C}/_{T}{}^{C}/_{T}$AA, containing the reverse complement of CCAAT (underlined). This sequence is common to a variety of eukaryotic genes including those of the mammalian major histocompatibility complex class II genes and vertebrate gamete-specific genes. All of the vertebrate Y-box proteins consist of 3 domains: a variable N-terminus; a highly conserved cold shock domain; and a hydrophilic C-terminal tail domain. In general, eukaryotic Y-box proteins function as transcriptional or translational regulators. For example, human YB1 acts as a transcriptional repressor. The following general observations were made for eukaryotic Y-box proteins (for a review see Wolffe, 1993):

- Y-box proteins preferentially interact with pyrimidine-rich rather than purine-rich ssDNA.
- Binding to duplex DNA occurs preferentially where the 2 strands of the double helix show separate enrichment for either purine or pyrimidine bases.
- The preference for purine or pyrimidine enrichment exceeds that of the Y-box region itself.
- Binding to the Y-box sequence is favoured over sequences in which the Y-box is mutated.
- Some, but not all, ssDNA sequences are favoured for binding over duplex DNA.

In addition, some vertebrate Y box proteins interact with RNA. Competition experiments demonstrated that *Xenopus* FRGY2 interacts with a variety of RNA molecules. In particular, this occurred with RNA which contained mixed polymers; poly (C, U), poly (A, C) and poly (A, U) in preference to homopolymers such as poly (A) (Wolffe, 1993).

1.6.3 The function of CspA-like proteins.

The cellular function of the CspA-like proteins has still to be determined fully. However, *in vitro* studies with *E. coli* CspA and *B. subtilis* CspB have shown that both proteins bind to ssDNA and dsDNA which contained the core sequence of the eukaryotic Y-box motif ATTGG, or its complement, in preference to DNA without the Y-box motif (Schindelin *et al.*, 1994, Graumann *et al.*, 1994). Interestingly, *in vitro* studies have shown that *E. coli* CspA enhances transcription of *gyrA* and *hns*, both of which are cold shock inducible proteins. Overexpression of *E. coli* CspA on a plasmid, compared to a wild type culture, was found to increase expression of at least 3 cold inducible proteins, including GyrA, following a downshift from 37°C to 15°C in a culture of exponentially growing cells (Jones *et al.*, 1992). Reporter studies with *E. coli hns::cat* translational fusion showed that addition of purified CspA protein to a coupled transcription-translation system resulted in an increase in CAT expression at both 37°C and 10°C (La Teana *et al.*, 1991). Furthermore, the *E. coli hns* contains the core Y-box sequence CCAAT, while the *E. coli gyrA* locus contains 3 copies of the reverse sequence ATTGG.

Purified *E. coli* CspA has been shown to bind to the regions of both *hns* and *gyrA* which encompasses the Y-box consensus sequence. In *E. coli hns*, the binding site was shown to be within a 110bp region carrying the *hns* promoter (La Teana *et al.*, 1991) and gel mobility shifts showed that purified *E. coli* CspA bound to a region within the promoter region of *E. coli gryA*, from -120 bp to +1 bp (Jones *et al.*, 1992). Indeed, CspA interaction with the promoter region of *gryA in vitro* declined in a proportional manner as each of the 3 ATTGG sites was mutated and DNA/protein interaction was abolished completely once all the Y-box sites were altered (Jones *et al.*, 1992). In contrast, the *Proteus vulgaris hns* locus does not contain the Y-box sequence, yet a DNA/protein complex was still formed with purified *E. coli* CspA and the *hns* DNA (Brandi *et al.*, 1994). Several *cspA* homologues, including *S. typhimurium cspA*, contain degenerate versions of the core Y-box sequence ATTGG. In the latter case, such a sequence lies upstream of the –35 promoter region.

Gel retardation studies have shown that purified *E. coli* CspA binds to ssRNA and ssDNA with approximately the same affinity. In both cases the molar concentration of CspA that caused the gel shift was approximately 2.7×10^{-5} M. Moreover, CspA/nucleotide binding appears to be co-operative, so that the protein/nucleotide complex band was supershifted above a concentration of 2.7×10^{-5} M CspA (Jiang *et al.*, 1997). The concentration of CspA in cells that have been cold shocked from 37°C to 15°C is estimated to be 10^{-4} M, which is almost 10-fold higher than the critical concentration observed during co-operative binding. Interesting, in this study, although CspA appeared to bind ssDNA, it was not reported to bind to dsDNA (Jiang *et al.*, 1997).

E. coli CspA has been shown to act as an RNA chaperone, enhancing the degradative activity of RNaseA and RNaseT1. An RNA secondary structure template that did not interact with CspA when in a denatured, single stranded form, was found to be more sensitive to degradation by RNaseA, in the presence of 3 μg CspA, when RNA secondary structures had formed (Jiang *et al.*, 1997). In addition, 142 nt RNA corresponding to the 5'-UTR of *E. coli cspA*, was similarly found to become more sensitive to degradation by RNaseA and RNaseT1 in the presence of purified CspA. These studies indicated that CspA stimulated RNase activity approximately 10-fold (Jiang *et al.*, 1997).

Studies with *B. subtilis* have shown that members of the cold shock family, CspB, CspC and CspD, are essential for cellular growth and for efficient protein synthesis at optimal and low temperatures (Graumann *et al.*, 1997). This group found that simultaneous deletion of all three cold shock proteins did not result in viable bacteria, when recombination events were attempted at 37°C. Formation of a triple mutant was only possible in the presence of *cspB* on a plasmid. Thus, CspA-like proteins of *B. subtilis* appear to play an essential role in survival at optimal growth temperature. Furthermore, the levels of CspB protein (detected by western blot) in the triple mutant that was complemented with *cspB* on a plasmid, were shown to be lower than from the wild type culture, following incubation at 15°C. In addition, growth of the

triple mutant that was complemented with *cspB* on a plasmid was shown to be greatly reduced at 15°C, relative to the wild type strain. Thus, synthesis of these cold shock proteins appears to be required for efficient adaptation to low temperatures in *B. subtilis* (Graumann *et al.*, 1997).

1.6.4 Regulation of the major cold shock protein.

An insight into the regulation of the major cold shock proteins has emerged following reasonably extensive studies into the regulation of *B. subtilis* CspB and especially *E. coli* CspA. The evidence of regulation of the major cold shock proteins will be discussed.

1.6.4.i Regulation at the level of gene transcription

The role of the UP element in cold shock regulation

It has been reported previously that a regulatory element, known as an UP element (an AT rich region) around –40 and –60 in some bacterial promoters, stimulates transcription by interacting with the α-subunit of RNA polymerase (Ross *et al.*, 1993). Similar AT rich regions are common to the promoter regions of several cold shock genes, including *E. coli cspA, cspB* and *csdA*. The *E. coli cspA* locus contains an UP element located between bases –60 and –40. Deletion analysis of this UP element resulted in a 4-fold decrease in β-galactosidase activity from a *cspA-lacZ* transcriptional fusion, following incubation at 15°C for 3 hours. This suggests that the UP element is essential for proper expression of CspA during cold shock (Mitta *et al.*, 1997). A number of *S. typhimurium cspA*-like genes have highly similar AT rich regions in the promoter region (this thesis, chapter 3, Craig *et al.*, 1998).

The role of the Cold Box in cold shock regulation

Studies on *E. coli cspA* has revealed the presence of an 11 bp motif, termed the cold box, located near the transcriptional start site, from bases +8 to +18. Further investigation has shown that the cold box consensus sequence UGACGUACAGA, is

also present in *cspB* and *csdA* (Jiang *et al.*, 1996). There is 1 mismatch between *E. coli cspA* and *csdA* and 2 mismatches between *E. coli cspA* and *cspB*. Mutational analysis of the *cspA* cold box sequence indicated that this region of DNA was responsible for down regulation of CspA and CsdA, which occurs after the acclimation phase (Fang *et al.*, 1997). Following incubation at 15°C for 3 hours, CspA and CsdA protein expression were maintained at a high level in the strain lacking the *cspA* cold box, relative to the wild type strain. RNA studies indicated that repression of chromosomal *cspA* occurs at the level of transcription and not at the level of mRNA stability (Fang *et al.*, 1997, Jiang *et al.*, 1996). The cold box consensus sequences of *S. typhimurium cspA* and *E. coli cspA* are 100 % identical. However, there are 3 mismatches and 1 deletion for *S. typhimurium cspB* (see figure 4.16 of this thesis).

The role of *E. coli* CspE in the expression of *cspA*.

Recent studies have shown that *E. coli* CspE may negatively regulate expression of *cspA* (Bae *et al.*, 1999). CspE is constitutively expressed at 37°C but is also transiently induced in lag phase. Interestingly, a culture of *E. coli* where the *cspE* ORF was replaced with the *kan* gene by homologous recombination, exhibited an increase of 2.5-fold in lag phase relative to the wild type strain. However, the growth rate exhibited following this lag was found to be similar to the wild type strain. Examination of protein synthesis of the *cspE* mutant strain, at 37°C, revealed several changes in protein profile including a 2-fold increase in production of CspA and a decrease in production of the universal stress protein, UspA. In addition, the synthesis of 3 other unidentified proteins increased and the synthesis of 6 proteins decreased. Moreover, analysis of a cell-free system (S-30 extract) showed that there was significant production of CspA at 37°C in the absence of CspE, whereas the production of CspA was inhibited in a proportional manner to the amount of purified CspE added. Analysis of *cspA* mRNA indicated that the inhibitory effect of CspE occurred at the level of transcription and not at the level of mRNA stability. Moreover, replacement of the *cspA* promoter region with the promoter for *lpp* indicated that CspE acts at the level of transcript elongation or termination and not at

the level of transcript initiation. Thus, CspE is thought to act as a transcriptional repressor of *cspA* (Bae *et al.*, 1999).

1.6.4.ii Regulation at the level of mRNA stability.

Differential stability of the *cspA* mRNA is thought to play a major role in the regulation of CspA. The mRNA of *E. coli cspA* is degraded extremely rapidly at 37°C and the half-life of the transcript has been estimated at approximately 10 seconds. However, a reduction in temperature stabilises the mRNA so that at 15°C the half-life of the transcript increases to 30 minutes (Goldenberg *et al.*, 1996). In parallel with the enhanced stability of *cspA* mRNA at low temperature, the level of *E. coli* CspA has also been shown to increase when the temperature was reduced from 37°C by as little as 6°C. Furthermore, the protein was produced at a high level between 25°C and 10°C (Etchegaray *et al.*, 1996).

Studies in *S. typhimurium* have demonstrated, by RNA analysis and protein fusion measurements, that *S. typhimurium cspB* mRNA is stabilised below a threshold temperature of approximately 22°C (Craig *et al.*, 1998). This contrasts to the situation proposed for *E. coli cspA* whereby it is the level of the temperature reduction that influences the amount of stable mRNA, but shows similarity to that of *E. coli cspB*. *E. coli cspB* mRNA appears to be most stable over a narrower temperature range than *E. coli cspA* mRNA, and was found to be most highly expressed at 15°C (Etchegaray *et al.*, 1996). However, activation also appeared to occur below a threshold temperature of approximately 24°C (Etchegaray *et al.*, 1996). Fusions of non-cold inducible genes, such as *lacZ*, to appropriate regions of the *E. coli cspA* promoter and 5'-UTR have been shown to stabilise *cspA* mRNA at 37°C, such that the half-life of the hybrid mRNA (-209 to +81 of *cspA*) was found to be 15 minutes, relative to a few seconds for the wild type *cspA* mRNA (Goldenberg *et al.*, 1996). The long 5' un-translated region of *E. coli cspA* contains a putative RNase E cleavage site, which is located near the translational start site. Disruption of this site also resulted in an increased half-life for the mRNA, to approximately 30 minutes (Fang *et al.*, 1997).

These findings strongly suggest that mRNA stability is fundamental to the cold shock induction of *E. coli* CspA and CspB, and of *S. typhimurium* CspB.

Studies by Bae and colleagues (1997) have shown that *E. coli* CspA appears to autoregulate its gene expression at the level of transcription and mRNA stability. Expression of the 5'-UTR was examined following a decrease in temperature from 37°C to 15°C, in a strain where the *cspA* ORF was deleted. The level of the 5'-UTR was found to be approximately 4 fold higher than observed in the wild type strain 30 minutes after the temperature shift to 15°C. In fact, increased production of the 5'-UTR was observed even after 7 hours of incubation at 15°C. In a similar manner, expression of the 5'-UTR of *E. coli cspB* was also examined following a temperature shift to 15°C. Although production of the *cspB* 5'-UTR was slightly higher than the wild type 5'-UTR (approximately 1.4-fold), following incubation at 15°C for 30 minutes, it appears that CspA specifically regulated expression of the *cspA* gene and only regulated expression of the *cspB* gene to a minor degree. The results were further supported by measuring reporter activity from a translational *cspA-lacZ* fusion on a low copy number plasmid, in a *ΔcspA* strain. In this case, *lacZ* was fused to the 13th codon of *cspA* and the level of β-galactosidase activity was found to increase approximately 2-fold relative to the wild type strain, after 2 hours incubation at 15°C. The level of increase of β-galactosidase activity was approximately 50% lower than the increase observed for the *cspA* 5'-UTR. This may, in part, be accounted for by other mechanisms of regulation.

Further studies were carried out to investigate the mRNA stability in the mutated *ΔcspA* strain, relative to the wild type strain. The stability of *cspA* mRNA was examined both 30 minutes and 3 hours after shifting a culture of exponentially growing cells to 15°C. The half-life of the transcripts from both the wild type strain and the *ΔcspA* strain was similar for the first 30 minutes after the temperature shift. However, after 3 hours incubation at 15°C, the half-life of the mRNA from the *ΔcspA* strain was approximately twice that of the wild type strain. It appears, therefore, that CspA negatively regulates its own gene expression at the level of transcription and at

the level of mRNA stability, although only at the end of the acclimation phase. In addition, *E. coli* CspA appeared to affect the level of other cold shock inducible CspA homologues. In the *ΔcspA* strain, the level of CspB and CspG was found to remain high for at least three hours relative to the level observed in the wild type strain, following a shift from 37°C to 15°C (Bae *et al.*, 1997).

1.6.4.iii Regulation at the level of translation.

Another factor that may be involved in up-regulation of cold shock gene expression is a region found in the coding region of *cspA* homologues, termed the downstream box (DB). It has been suggested that the DB interacts with the complementary sequence of 16S rRNA that mediates efficient translation from the initiation codon, independently of the RBS (Sprengart *et al.*, 1996). In *E. coli cspA*, the DB encompasses the first 10 codons of the gene and has a high level of complementarity to the anti-DB of 16S rRNA (see figure 4.2 of this thesis). Mutational analysis, where part of the DB of *E. coli cspA* was deleted, resulted in a 50% reduction in β-galactosidase activity from a *cspA-lacZ* translational fusion (Mitta *et al.*, 1997).

1.6.5 The role of ribosomes in regulation of the cold shock response.

There has been some evidence to suggest that the physical state of the ribosome may act as a sensor which initiates the cold shock response under appropriate conditions. This data is presented below.

1.6.5.i Role of guanosine nucleotides in the cold shock response.

A number of studies in *E. coli* have indicated that the concentration of low molecular weight solutes, AppppA, pppGpp and ppGpp - known as alarmones - may be central to the regulation of stress responses such as the heat shock, oxidative shock and cold shock. Induction of the stringent response, which arises because of amino acid starvation, results in the accumulation of pppGpp (guanosine 5'-triphosphate-3'-

diphosphate) and ppGpp (guanosine 5'-diphosphate-3'-diphosphate), collectively known as (p)ppGpp. When the ratio of charged (aminoacylated) to uncharged tRNA falls, the requirement for protein synthesis cannot be met, which causes the ribosome to stall during translation. Under these conditions, the stalled ribosome, to which the *relA* product (encoding (p)ppGpp Synthetase I) is bound, binds uncharged tRNA, releases it and stimulates (p)ppGpp synthesis. In addition, a *relA* independent pathway for (p)ppGpp synthesis exists which involves *spoT*, encoding (p)ppGpp Synthetase II. This system is activated during carbon starvation, but is also responsible for (p)ppGpp degradation by the enzyme (p)ppGpp 3'-pyrophosphohyrolase during the stringent response (Cashel & Rudd, 1987). Rudd *et al.* (1985) demonstrated that *E. coli* and *S. typhimurium spoT* mutants exhibit (i) elevated levels of (p)ppGpp during slow growth, (ii) a higher level of (p)ppGpp during amino acid deprivation, (iii) a slower decay of (p)ppGpp when amino acid starved cells are re-supplemented with amino acids, and (iv) failure to accumulate (p)ppGpp during amino acid starvation.

In *E. coli*, an increase in the level of the (p)ppGpp occurs in nutrient downshift conditions and results in a co-ordinated decrease in the rate of synthesis of RNA and ribosomal proteins (Pao & Dyess, 1981). Under these conditions, the synthesis of translational components that are in functional excess, is decreased, and the synthesis of enzymes of biosynthetic and fuelling pathways, is increased. Changes in the (p)ppGpp level also occur in response to temperature changes (Jones *et al.*, 1992). However, these changes have not been as extensively characterised as the effects of nutritional changes. Nevertheless, the level of (p)ppGpp is known to decrease in a proportional manner to temperature downshift (Pao & Dyess, 1981).

Cold shock results in an immediate decrease in total RNA synthesis, followed by a decrease in the (p)ppGpp level and a corresponding increase in the rate of synthesis of RNA (Jones *et al.*, 1992). During the 4 hour lag that follows shifting the cells from 37°C to 10°C, the decrease in the (p)ppGpp level appears to positively correlate with the synthesis of some transcriptional and translational proteins, such as NusA, Pnp

and IF2, and many cold shock proteins. Exposing an exponential culture of *E. coli* to a nutritional downshift 10 minutes prior to a temperature downshift to 10°C for 4 hours, resulted in repression of NusA, PNP, the dihydrolipoamide acetyltransferase subunit of pyruvate dehydrogenase (AceE), pyruvate dehydrogenase (lipoamide) and CspA. In addition, artificial overproduction of (p)ppGpp using a *p-tac*::*relA* fusion, and incubation of the culture at 15°C for 10 minutes, resulted in severe reduction in synthesis of a number of proteins, including CspA. The synthesis of H-NS was not affected, while the synthesis of DnaK and GroEL - normally repressed by the cold shock response - was found to increase. In contrast, a *relAspoT* double mutant which is essentially (p)ppGpp-free, exhibited a high level of CspA at 24°C (Jones *et al.*, 1992).

Overall, these studies suggest that the cold shock response can be likened to a nutritional upshift in terms of insufficient capacity for protein synthesis relative to the supply of charged tRNA. It has been proposed that the physiological trigger that results in a drop in the level of (p)ppGpp is the decreased translational capacity of the cell which in turn correlates with the changes in gene expression. Thus, (p)ppGpp appears to act as a modulator of the cold shock response, or at least inversely correlates with the cold shock response (Jones & Inouye, 1994).

1.6.5.ii The effects of antibiotics on the cold shock response.

2-dimensional gel electrophoresis studies have suggested that the cold shock and heat shock responses can be induced, to an extent, by exposure to sub-lethal concentrations of antibiotics that interfere with ribosome action. The heat shock inducing, or H-antibiotics include kanamycin, puromycin and streptomycin. The cold shock inducing, or C-antibiotics include chloramphenicol, erythromycin, fusic acid, spiramycin and tetracycline. The heat and cold shock simulated responses were demonstrated by adding the antibiotics to exponential cultures at 37°C. Addition of any of the C-group of antibiotics resulted in induction of 10 cold shock proteins and almost complete repression of heat shock protein synthesis. In addition, a slight

increase in the synthesis of ribosomal proteins and other stringently controlled factors occurred, and, as with the cold shock response, (p)ppGpp levels decreased (VanBogelen and Neidhart, 1990).

Thus, preliminary studies have provided evidence that the ribosome may act as a temperature sensor where the physical status of the ribosome, *per se*, or some of its products, provide a signal linking the current temperature to the cellular response. In line with this, Jones *et al.* (1992) have suggested that a sharp downshift in temperature induces a physiological state where the translational capacity of the cell is insufficient relative to the supply of charged tRNAs. This then signals a decrease in the level of (p)ppGpp and the induction of the cold shock response.

1.7 AIMS OF THIS STUDY

1.7.1 Background

Overall, this project aims to explore the molecular basis of the cold shock response of an important food pathogen, *S. typhimurium*, in conditions which equate with those used in industrial food storage and food processing. Previously Francis (1993) identified a *S. typhimurium* cold-inducible locus, using a Mu*dlux* transcriptional reporter system. Further studies demonstrated that this gene was similar to *E. coli cspA* and later termed *cspB*. Expression of *cspB* was both highly and rapidly induced following incubation of exponentially growing cells at low temperatures (Craig *et al.*, 1998). In addition, a second cold inducible gene was located on the *S. typhimurium* chromosome, which encoded CspA, a protein identical in amino acid sequence to the major cold shock protein of *E. coli* CspA (Craig & Gallagher, unpublished). The effects of growth phase on the cold shock response were investigated, following the extensive body of work on the increased resistance of stationary phase cells to environmental stresses.

1.7.2 Project aims

- Investigation of the role of the major cold shock protein, CspA. For this, attempts were made to construct a strain in which *cspA* was disrupted by replacing the coding region with an antibiotic resistance gene. Previous studies with *E. coli* and *B. subtilis* have shown that changes in the proteome and in growth rate occur, when one or more of the cold inducible cold shock genes have been disrupted (Bae *et al.*, 1997, Graumann *et al.*, 1997).
- Expression of cold shock inducible *cspA* homologous genes were investigated in *S. typhimurium*. Expression of *cspA* was monitored using a transcriptional *cspA-lacZ* fusion Expression of a second CspA homologue, CspB, was explored by monitoring reporter activity from a chromosomal translational *cspB::lux* fusion. Extensive studies on the alternative sigma factor, σ^S, have shown that it plays an important role in stress responses of *E. coli* (Loewen and Hengge-Aronis, 1994). The effect of σ^S on the expression of *cspB* was examined at low temperatures. Studies on *E. coli* Fis have shown that this protein is highly induced following entry into lag phase (Osuna *et al.*, 1995). The cold shock response has been likened to a nutritional up-shift in terms of the state of the ribosome, therefore the potential role of Fis was also investigated at low temperatures.
- The effect of rapidly chilling cultures of *S. typhimurium* was investigated and survival was measured in terms of plating ability. Studies with *E. coli* have shown that survival at 4°C is very low during early and mid exponential phases of growth, but that survival is almost complete when the cells enter stationary phase (Meynell, 1958). Survival of different strains was monitored following dilution into a salt solution at 4°C.
- Changes in the proteome were monitored by 2D PAGE, in both exponentially growing and stationary phase cells, and following shifts to temperature just above and just below the minimum temperature permissible for growth. Changes in the proteome are routinely used to investigate stress responses of bacteria. Investigation of protein synthesis by an important pathogen such as *S.*

typhimurium, at refrigerated temperatures, is fundamental to understanding the molecular basis of the cold shock response.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Bacterial strains Plasmids and Oligonucleotides.

Bacterial strains and plasmids and their sources are shown in tables 2.1 and 2.2.

Table 2.1 Bacterial strains used in this thesis.

Bacterial Strain	Genotype	Reference/ Source
Escherichia coli		
EMG2	Wild type	
DH5α	*supE44 ΔlacU169* (ϕ80 *lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan (1985)
MM38	*pcnB::kan*	Masters, M. (1993)
Salmonella typhimurium		
SL1344	*his*$^-$	Hosieth and Stocker (1981) S.G.S.C.
LT2	Wild type	S.G.S.C.
CH607	*polA*$^-$	S.G.S.C.
RJ1829	*fisΔ cat*	S.G.S.C.
AK3312	*pyrE27 oxrE11*::Tn10	S.G.S.C.
AK3040	*xylA26*::Tn10	S.G.S.C.
AK3294	*gltC*::Tn10	S.G.S.C.
TT7247	*zbe-1023*::Tn10	S.G.S.C.
TN1744	*arg88 apeA18 apeB21 zbc-873*::Tn10	S.G.S.C.
MPG361	*cspB*::Mu*dlux*, SL1344 background	This lab (Francis, K.P.).
MPG480	*rpoS:: bla*, SL1344 background	This thesis
MPG481	*fisΔ cat*, SL1344 background	This thesis
MPG490	*recD*::Tn10 (*tet*), *hsdLT, hsdSA, hsdSB*	This lab (Gallagher, M.P.).
MPG491	derivative of AK3312 *	This thesis
MPG492	derivative of AK3040 *	This thesis

Table 2.1 continued.

MPG493	derivative of AK3294 [a]	This thesis
MPG494	derivative of TT7247 [a]	This thesis
MPG495	derivative of TN1744 [a]	This thesis

All strain were grown at 37°C, unless otherwise indicated. (a – the indicated strains were provided by the Salmonella Genetic Stock Centre (S.G.S.C.) (Kukral *et al,* 1987).

Table 2.2 Plasmids and their derivatives used in this thesis.

PLASMIDS	RELEVANT FEATURES	SOURCE / REFERENCE
pBR322	pMB1 relpicon Amp^R, Tet^R	Bolivar *et al.*, (1977)
pBR325	pMB1 replicon, Amp^R, Tet^R, Cml^R	Bolivar *et al.*, (1978)
pBluescript SK (M13+)	ColE1 – like relpicon Amp^r carries *lacZ*α containing a multiple cloning site	Short *et al.*, (1988)
pUC19	General cloning vector, contains MCS of M13mp7	Vieira & Messing (1982)
pBRX	A modified pBR322 plasmid where the *Hind*III was removed and replaced with *Xba*I	This thesis
pJEC17	pKS derivative containing 4.0 Kb *EcoRI* fragment which includes the *S. typhimurium cspA* gene.	J.E. Craig, unpublished
pLJF1	pKS derivative in which the *cspA* ORF and *cspA* P regions were removed from pJEC17, by digestion with *Hind*III.	L.J. Foster unpublished
pNJH1	pKS derivative in which the *cspA* P region, amplified by PCR using primers MICG1 and MICG2, was introduced into the *Sal*I and *Hind*III sites of pLJF1.	This thesis.
pNJH5	pBR322 derivative containing an *Xba*I fragment which includes the *cspA* P region and region downstream of *cspA*, sub-cloned from pNJH1, into the *Xba*I site in pBRX	This thesis.
pNJH6	pBR322 derivative containing the *lacZ* gene, amplified by PCR using primers LAC5 and LACZR, that had been inserted into the *Bgl*II and *Hind*III sites in pNJH5.	This thesis.
pNJH7	pBR322 derivative containing the *cml* cassette, amplified by PCR using primers 5'-CML and 3'-CML, that had been inserted in to the *Hind*III site in pNJH6.	This thesis.
pNJH8	pBR322 derivative containing a promoter-less *cml* gene, sub-cloned from pCM4 and inserted in the *Bgl*II site in pNJH1.	This thesis.
pNJH9	pBR322 derivative containing a *cml* cassette, amplified by PCR using primers 5'-CML and 3'-CML, that had been inserted in the *Hind*III site in pNJH1.	This thesis.

Table 2.2 continued

pNJH10	pBR322 derivative containing an *Xba*I-*Pst*I fragment that includes the *cspA* P region, the *cat* cassette and the region downstream of *cspA*, sub-cloned from pNJH5 into the corresponding sites in pBRX.	This thesis.
pNJH12	pUC19 derivative that contained the *cspA* P region, the *cat* cassette and the region downstream of *cspA*, amplified by PCR using primers MICG1 and CSPA6, inserted into the *Sal*I site in pUC19.	This thesis.
pCM4	Contains the promoterless *cat* gene, flanked by 2 BamHI sites.	Close & Rodrigez, 1982
pPDT11	pBR322 derivative containing *ahp* that had been disrupted by insertion of a *cat* cassette.	Taylor *et al.*, 1998
pAHP	pUC19 derivative that contained *ahp-cat* fragment, amplified by PCR using primer G7858 and AHP3, inserted into the *Bam*HI and *Nde*I sites in pUC19	This thesis

Synthetic oligonucleotides were purchased from Perkin Elmer Ltd. (Cheshire, UK). The oligonucleotides used in this study are listed in table 2.3.

PRIMER No.	PRIMER SEQUENCE	COMMENTS AND USE
MICG1 (1 – 23)	5' - AGGTCGACGGTCTAGAGCTTGATATCGAAT *SalI* *XbaI* TCGCCAGAGCGCTGGGCGTAACGG - 3'	PCR of *S. typhimurium cspA* promoter region (5'-3').
MICG2 (376 – 336)	5' - GCAAGCTTTACAGATCTTTAAAGCGTTAAG *HindIII* GAAATGCGCACTACGAGGGG T - 3'	PCR of *S. typhimurium cspA* promoter region (3'-5').
CSPA6 (1142 – 1119)	5'- ACGGTCGACTCGGCATTTCTCCC – 3' *SalI*	PCR of *S. typhimurium cspA* downstream region (3'-5').
CSPA1 (576 – 594)	5'- CCTTCACCATCGAAAGCGG – 3'	Sequencing of *S. typhimurium cspA* gene (5'-3')
5'-CML (4288 – 4312)	5' – CGAAGCTTAAACCATTATTATCATG – 3' *HindIII*	PCR of PBR325 *cat* cassette (5'-3').
3'-CML (5262 – 5234)	5' – CGAAGCTTAGATCTCAGGCGTTTAAGG *HindIII* *BglII* GCACCAATAACTGCCT – 3'	PCR of PBR325 *cat* cassette (3'-5').
LAC5 (1232 – 1262)	5' – TCAGATCTGTGAGCGGATAAC – 3' *BglII*	PCR of *E. coli lac* operon (5'-3').
LACZR (4367 – 4340)	5' – CGAAGCTTATTATTTTTGACACAAGACC – 3' *HindIII*	PCR of *E. coli lacZ* (3'-5').
G7858 (198-214)	5' – GCGGATCCCAAAAACCAGGCGTTCA – 3' *BamHI*	PCR of *S. typhimurium ahpC* promoter region (5'-3').
AHP3 (2093 - 2069)	5' – CCAGATCCATATGCGATTCAACGC – 3' *NdeI*	PCR of *S. typhimurium ahpC* downstream region (3'-5').

Table 2.3 Oligonucleotides used in this study. The sequence of the oligonucleotides (primers) that were used in this study is shown. The numbers in parentheses refer to the position of the published DNA sequence to which the primers corresponded. The positions of the relevant restriction enzyme sites have been underlined, and the names given. *cspA* (J.E. Craig & L. Chamberlin, unpublished), *ahpCF* (Tartaglia *et al.*, 1990); *cat* (Bolivar *et al.*, 1988), *lac* operon (Kalnins *et al.*, 1988).

2.1.2. Enzymes, isoptes and chemicals.

Enzymes were purchased from Boehringer Mannheim UK (Diagnostics and Biochemicals) Ltd., Bell Lane, East Sussex, unless otherwise stated. Stabilised aqueous solutions of α- ^{32}P – dCTP (3000Ci/mM; 10μCi/ml) were purchased from Amersham International plc., Lincoln Place, Buckinghamshire. Chemicals: standard agarose was supplied from Northumbria Biologicals Ltd., Nelson Industrial Estate, Cramlington, Northumberland; all antibiotics were purchased from Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset; general laboratory chemicals were purchased from Sigma Chemical Company, BDH Chemiclas Limited, Merck House, Poole, Dorset and Fisons Scientific Equipment, Bishop Meadow Road, Leicestershire. Nylon filters were purchased from BIO – RAD Laboratories Inc., Maylands Avenue, Hemel Hampstead, Hertfordshire. X – ray film was purchased from AGFA (H. A. West, Edinburgh).

2.1.3. Solutions

All solutions were made up in dH_2O and sterilised by autoclaving at 15 pounds per square inch for 20 minutes prior to use. Heat labile components were separately filter sterilised before they were added to the appropriate main solution, that had been previously sterilised by autoclaving.

Tris:HCl: 1 litre contained Tris base (tris [hydroxymethyl] aminomethane) dissolved to the desired molarity in dH_2O and the pH was adjusted to the required value by addition of concentrated HCl.

EDTA: 1 litre contained EDTA (ethylenediaminetetraacetic acid, di-sodium salt) dissolved in dH_2O to a concentration of 0.5 M and was adjusted to pH 8.0 by addition of NaOH.

TE buffer: 1 litre contained 10 mM Tris base and 1 mM EDTA (disodium salt) dissolved in dH_2O and adjusted to the appropriate pH with HCl.

10 x TBE: 1 litre contained 0.9 M Tris base, 0.9 M boric acid and 0.02 M EDTA (disodium salt) dissolved in dH_2O.

20 x SSC: 1 litre contained 3 M NaCl and 0.3 M tri-sodium citrate dissolved in dH_2O.

Phenol::Chloroform Equal volumes of pre-equilibrated phenol pH 8.0 (Sigma) and chloroform were added to an air-tight bottle. The solution was aliquoted in 10 ml portions and overlaid with 5ml of 0.1 M Tris:HCl pH 8.0 and stored at 4°C.

Sodium acetate: 1 litre contained sodium acetate dissolved in dH_2O to a final concentration of 3 M and the pH adjusted to 5.0 with acetic acid.

Ethidium bromide contained ethidium bromide dissolved in dH_2O as a stock solution of 10 mg ml^{-1} and stored at room temperature in the dark.

6x loading buffer for nucleic acid gel electrophoresis contained 15% (w/v) Ficoll with 0.25 % (w/v) bromophenol blue and 0.25 % (w/v) xylene green.

25% Ringers solution:1 litre contained 2.5 g NaCl, 0.05 g $CaCl_2.2H_2O$, 0.05 g KCl, 0.025 g $NaHCO_3$ and 0.25 g glucose. The solution was aliquoted into 100 ml portions and autoclaved.

Z buffer: 1 litre contained 16.1 g $Na_2HPO_4.7H_2O$, 5.5 g $NaH_2PO_4.H_2O$, 0.75 g KCl, 0.246 g $MgSO_4.7H_2O$ and 2.7 ml β- mercaptoethanol, adjusted to pH 7.0 and stored at 4°C.

Recipes for all other buffers are included in the appropriate methods section.

ANTIBIOTIC	SOLVENT	STOCK CONCENTRATION	FINAL CONCENTRATION
Amplicillin	dH_2O	10 mg ml^{-1}	50 μg ml^{-1}
Chloramphenicol	Ethanol	25 mg ml^{-1}	25 μg ml^{-1}
Kanamycin	dH_2O	10 mg ml^{-1}	50 μg ml^{-1}
Tetracycline	50% Ethanol	10 mg ml^{-1}	10 μg ml^{-1}

Table 2.4 Antibiotics used in this thesis.

2.1.4. Molecular weight standard markers.

Molecular weight standard markers (normal and pre-stained) for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of proteins are indicated in table 2.5, table 2.6 and 2.7.

Protein Standard	Approximate Molecular Weight (Da)
Myoglobin (Polypeptide backbone 1 – 153)	17, 000
Myoglobin (I + II, 1 – 131)	14, 400
Myoglobin (I + III, 56 – 153)	10, 600
Myoglobin (I, 56 – 131)	8, 200
Myoglobin (II, 1 – 55)	6, 200
Glucagon	3, 500
Myoglobin (III, 132 – 153)	2, 500

Table 2.5 Molecular weight markers. Markers (MW-SDS-17S, Sigma) were used as a mixture of standards for tricine buffered polyacrylamide gels.

Protein Standard	Approximate Molecular Weight (Da)	Approximate pI
Amyloglucosidase	70, 000	3.8
Ovalbumin	45, 000	5.1
Carbonic Anhydrase	29, 000	7.0
Myoglobin	17, 000	7.6

Table 2.6 2-D electrophoresis markers. Markers (A8437, O4757, C4806, M7911, Sigma) were used as a mixture for 2-D PAGE.

Protein Standard	Native Molecular Weight (Da)	Apparent Molecular Weight (Da)
Pyruvate Kinase	58, 000	75, 200
Triosephosphate Isomerase	26, 600	35, 200
Aprotinin	6, 000	7, 000

Table 2.7 Pre-stained molecular weight markers used for polyacrylamide gels. Markers (P5788, T9400 and A2315, Sigma) were used as a mixture of standards for SDS-PAGE. Aprotinin is a fluorescent conjugate, pyruvate kinase and triosephosphate isomerase are conjugated to a blue dye.

2.1.5. Bacterial Media.

Luria Bertani (LB) broth contained Difco Bacto tryptone (10 g), Difco Bacto yeast extract (5 g) and NaCl (10 g) dissolved in 1 l dH_2O. The pH was adjusted to 7.2 with 5 N NaOH and the media autoclaved (20 lb/in^2, 120°C for 15 minutes).

Nutrient Broth (NB) contained Difco Bacto casamino acids (42.4 g), Difco Bacto yeast extract (5.2 g), NaCl (27 g) and K_2HPO_4 (10.4 g) dissolved in 1 l dH_2O prior to autoclaving as above.

Solid Agars LB and NB were converted to solid media by adding Difco agar (15 g l^{-1}) prior to autoclaving.

Soft (Top) Agars contained 0.6 % (w/v) agar added to the appropriate medium prior to autoclaving.

SOC medium contained Difco bacto-tryptone (20 g), Difco yeast extract (5 g), NaCl (10 mM), KCl (2.5 mM), $MgCl_2$ (10 mM), $MgSO_4$ (10 mM) and glucose (20 mM) dissolved in 1 litre of dH_2O. The solution was sterilised by autoclaving.

Spitzizen minimal medium (Spitzizen, 1958) contained 300 ml dH_2O, 80 ml (4x) Spitzizen Salts (7 g K_2HPO_4, 10 g $(NH_4)SO_4$, 3 g KH_2PO_4, 5 g sodium citrate and 1 g $MgSO_4.7\ H_2O$ dissolved in 1 litre of water), 10 ml 20% (w/v) glucose and 0.5 ml thiamine B1 (1 mg ml^{-1}).

2.2. METHODS

2.2.1. Manipulation of bacteria and phage.

Growth of bacterial cultures.

Overnight liquid cultures of *E. coli* and *S. typhimurium* were prepared by inoculating 5 ml of LB with a single colony. Cultures were grown with shaking at 37°C. Larger cultures were prepared by diluting overnight cultures as required (1 : 50 or 1 : 100) in conical flasks with a total capacity 5 to 10 times that of the final culture volume. The cultures were grown as above.

Other bacteria were grown similarly in the appropriate growth medium at the appropriate temperature (table 2.1).

Storage of bacterial cultures.

For long term storage, 1 ml of fresh overnight culture of bacteria, grown in rich media supplemented with antibiotic if necessary, was mixed with 70 µl of 100 % DMSO and stored in a sterile vial at –70°C. Cultures were recovered by using sterile sticks to transfer bacteria to appropriate agar plates with antibiotic, if required. After overnight incubation at the appropriate temperature, a single colony was picked to propagate a fresh bacterial culture. For short term storage (up to 4 weeks), bacteria were stored as streaks on agar plates at 4°C.

Preparation of indicator cells.

A fresh overnight culture of the appropriate host was diluted 100 fold into LB and grown at 37°C to late log phase ($O.D._{600nm}$ = 0.6 to 1.0). 100 µl of indicator cells were added to 3 ml of top agar for P22 lysates.

Amplification of P22 lysates.

A fresh overnight culture was diluted into 50 fold into 10 ml LB and grown at 37°C for 45 minutes. Phage were added to give a multiplicity of infection (m.o.i.) of 1.0. The culture was incubated for 8 hours at 37°C, during which lysis was observed to occur, 0.2 ml $CHCl_3$ was added and the culture was vortexed before storing at 4°C for at least 2 hours. Cell debris was pelleted in a bench top centrifuge (MSE Centaur 1) for 5 minutes at 3,000 g and the partially cleared lysate transferred to a sterile bottle. 0.2 ml $CHCL_3$ was added, the solution vortexed and stored at 4°C for at least 2 hours. The solution was centrifuged again for 5 minutes at 3,000 g and the cleared lysate transferred to a sterile bottle. The lysate was stored with 0.2 ml $CHCl_3$ at 4°C and was stable for several months.

Phage titration.

Serial dilutions of the test phage were made in LB and 100 µl of each dilution added to indicator cells (described above). 3 ml of molten top agar was added and the suspension poured onto fresh LB plates. Plaques were counted and the titre determined. Generally P22 lysates had a titre of 10^9 ml^{-1}.

2.2.2. Nucleic acid manipulation and detection methods.

Agarose gel electrophoresis.

DNA was resolved by agarose gel electrophoresis. The gel consisted of electrophoresis grade agarose (0.8 % to 1 % w/v in 1x TBE) containing 0.5 µg ml^{-1} ethidium bromide. DNA was prepared for loading by adding $1/6^{th}$ of the total volume of 6x loading buffer and electrophoresed at 100 V, in a 110 mm x 150 mm horizontal gel unless otherwise indicated, until suitable resolution had been obtained. Nucleic acid size was estimated in relation to the position of pre-digested *Hind*III phage Lambda DNA fragments (from

Boehringer Mannheim) in the agarose gel. DNA was visualised under UV light (λ=313 nm) and photographed using a Mitsubishi video copy processor.

PCR

The polymerase chain reaction (PCR) was used for amplification of DNA fragments. Primers specific for the region of DNA of interest were designed as required (Table 2.3 lists the primers used in this study). Reactions were performed in 100 µl volumes containing template DNA (10 ng), 2.5 µl of each primer (100 pM), 10 µl of 10x PCR reaction buffer (Boehringer Mannheim), 8 µl of dNTP mix (containing 2.5 mM of each dNTP), 1 µl Taq DNA polymerase (2 U μl^{-1} Boehringer Mannheim) and dH_2O. 100 µl of sterile mineral oil was added to the surface of the reaction mixture to prevent evaporation during the PCR process. The PCR was carried out in a Techne 'Gene E' thermal cycler dry-block.

Amplification of the DNA was performed over a total of 25 cycles, each cycle consisted of the following three steps: Denaturation at 94°C for 1 minute, annealing at 50°C to 55°C (dependent of the length and nucleotide content of the primer) for 1 minute and elongation at 72°C for 2 minutes. After the end of the 25 cycles, a final single cycle of 72°C for 10 minutes was performed to complete the process. PCR reaction mixtures were then examined under agarose gel electrophoresis.

Phenol chloroform equilibration.

Pre-equilibrated phenol (Sigma) was prepared by the addition of 0.1% (w/v) 8-hydroxyquinoline, followed by an equal volume 0.1 M Tris. Cl, pH 8.0 and stored in 10 ml aliquots at 4°C.

Phenol - chloroform extraction of DNA.

Phenol - chloroform extractions were performed to remove cell debris and proteins associated with DNA. An equal volume of equilibrated phenol was added to one volume of a DNA solution, normally 200 µl, and the solution was mixed vigorously with vortexing. The aqueous phase (containing the DNA) and the phenolic phase were separated by centrifugation at 20,000 *g* for 2 minutes. The aqueous layer was then transferred to a fresh micro-centrifuge tube and 1 volume of $CHCl_3$ was added. The two phases were mixed and separated as before and the process was repeated. Finally, the aqueous layer was transferred to a fresh tube, leaving a small amount to prevent carry-over of the chloroform phase.

Ethanol precipitation

In a micro-centrifuge tube, 0.1 of the volume of sodium acetate (3 M, pH 5.0) was added to 1 volume of DNA, followed by 2.5 times the total volume of absolute ethanol. The solution was mixed thoroughly by inverting and incubated at -80°C for 15 minutes, after which the DNA was pelleted by centrifugation at 20, 000 *g* for 15 minutes. The pellet was then washed with 1 volume of 70% ethanol to remove salt and centrifuged for a further 5 minutes at 20, 000 *g*. The pellet was subsequently dried under vacuum and resuspended in dH_2O or TE.

Restriction endonuclease digest.

0.1-20 µg of DNA was digested in dH_2O containing 0.1 volume of a 10x enzyme buffer (appropriate to the enzyme) and enzyme was added according to the manufacturers guidelines (normally 1 unit of enzyme per 1 µl of DNA). Digestion was allowed to proceed for 1-2 hours at 37°C (unless the manufacturer suggested otherwise) and overnight for chromosomal DNA. For double digests involving enzymes with different

recommended buffers, DNA was first cleaved with the enzyme requiring the lowest salt buffer, after which the digest was optimised for the higher salt buffered enzyme.

De-phosphorylation of vector.

Plasmid vectors digested with restriction enzymes were treated with shrimp alkaline phosphatase (SAP) to aid cloning of inserts into the vector. The cut vector DNA was incubated at 37°C for 1 hour in dH_2O containing 0.1 volume of 10x SAP buffer and 1 unit of SAP. The SAP was then inactivated by incubation at 65°C for 20 minutes and the DNA was purified by phenol - chloroform extraction, followed by ethanol precipitation.

Klenow enzyme.

DNA that had been digested with restriction nucleases that resulted in either a 3' or 5' termini overhang, were end-filled with the Klenow fragment of DNA polyermase I, to generate blunt ended termini. 0.1-20 μg of DNA was incubated with 2 U of Klenow enzyme and 10 pmol of dNTPs for hour at 37°C. The DNA was then purified using the phenol - chloroform method, followed by ethanol precipitation.

Ligation.

Ligations were carried out in dH_2O containing 0.1 volume of 10x ligase buffer, 5 units of T4 DNA ligase (Boehringer Mannheim) and DNA vector (50-100 ng) in a final volume of 10 μl. The reaction mixture was incubated overnight at 15°C with 3 times the molar ratio of DNA insert.

Large scale extraction of *E. coli* plasmid DNA.

To obtain large quantities of very pure plasmid DNA, the protocols of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981) were employed.

Bacterial cultures harbouring the plasmid of interest were grown overnight in 500 ml of LB media containing the appropriate antibiotic(s), at 37°C with shaking. Cells were pelleted at 4, 000 *g* for 10 minutes in a Beckman J2-21 centrifuge (JA-14 rotor) and excess liquid was removed by inversion of the centrifuge container for 5 minutes. The pellet was resuspended in 18 ml of Solution I (50 mM glucose, 25 mM Tris. Cl, pH 8.0 and 10 mM EDTA) and was then treated with 1 ml of a 10 mg ml^{-1} lysozyme solution for 5 minutes at room temperature. 40 ml of Solution II (0.2 N NaOH and 1% [w/v] SDS) was then added, the solution was mixed by inversion several times and incubated on ice for 5 minutes. After this time, 20 ml of ice-cold Solution III (5 M potassium acetate buffer, pH 4.8) was mixed thoroughly with the sample. The sample was incubated on ice for 15 minutes and was then centrifuged at 8, 000 *g* for 5 minutes at 4°C to pellet the cell debris. Unpelleted cell debris was removed by filtering the supernatant through non-absorbent cotton wool, into a fresh centrifuge container. 48 ml isopropanol was added and the sample was inverted, gently 3 times. The plasmid DNA was harvested by centrifugation at 10, 000 *g* for 5 minutes, the pellet was rinsed with 70% (v/v) ethanol, the centrifuge container was inverted to remove excess liquid and the pellet was air dried. The dried pellet was then resuspended TE to a final volume of 9 ml and 9.2 g of CsCl was added, followed by 0.25 ml of a 10 mg ml^{-1} ethidium bromide solution. The sample was mixed by inversion and then centrifuged in a MSE Centaur I centrifuge at 4, 000 *g* to remove precipitates. 1 ml of the cleared supernatant was weighed to ensure the correct density of 1.55 g ml^{-1}. The sample was then transferred to Sorvall TV865 quickseal ultracentrifuge tubes and centrifuged at 200, 000 *g* for 18 hours at 18°C in a Sorvall OTD55B ultracentrifuge. The plasmid band was visualised on the CsCl gradient under UV light, and was extracted using a sterile needle and syringe. Ethidium bromide was removed by mixing the sample, at least 4 times, with TE

saturated butan-1-ol, discarding the upper (butanol/ethidium bromide) layer each time. Extraction of ethidium bromide was complete when the lower, aqueous layer, containing the plasmid DNA was completely clear. The aqueous phase was then dialysed against TE several times to remove the CsCl. Subsequently, the DNA solution was examined by agarose gel electrophoresis, and if necessary, concentrated by ethanol precipitation.

Small scale preparation of *E. coli* and *S. typhimurium* plasmid DNA.

E. coli and *S. typhimurium* cells harbouring the plasmid of interest were grown overnight in 5 ml LB containing the appropriate antibiotic, at 37°C with shaking. The plasmid DNA then was prepared by the alkali lysis method, which was essentially the same method employed for the large scale extraction of plasmid DNA and used the same solutions. Cells were pelleted in a micro-centrifuge tubes at 20, 000 *g* for 40 seconds. The supernatant was removed and 200 µl of Solution I was added, as well as 10 µl of a 10 mg ml^{-1} lysozyme solution. After mixing by inversion, the tube was left to stand for 2 minutes. 300 µl of Solution II was then added and the sample was mixed thoroughly by inversion. 300 µl of Solution III was finally added, the solution mixed as before and incubated on ice for 5 minutes. The cell debris was removed by centrifuging the sample at 20, 000 g for 10 minutes and the supernatant was transferred to a fresh micro-centrifuge tube. RNaseA (DNase-free) (Boehringer Mannheim) was added to a final concentration of 20µg ml^{-1} and the solution was incubated at 37°C for 20 minutes. The DNA was then extracted with 400 µl chloroform and the DNA was precipitated by adding an equal volume of isopropanol. The DNA was pelleted by centrifugation at 20, 000 *g* for 10 minutes immediately after the isopropanol was added. The pellet was rinsed with 70 % (v/v) ethanol, dried and resuspended in dH_2O.

Transformation of plasmid DNA by electroporation.

The majority of transformations were carried out with DH5α as the host strain, unless other wise stated. A single colony of the appropriate strain from a fresh LB plate was inoculated into 5 ml LB containing the appropriate antibiotic(s) and grown overnight at 37°C with shaking. The cells were diluted 100 fold into 500ml LB and grown until the $O.D._{600}$ reached 0.5. The cells were harvested by centrifugation and resuspended in 200 ml sterile dH_2O. The cell suspension was incubated on ice for 1 hour. The cells were washed 3 times in 200 ml sterile ice cold dH_2O, each time by centrifugation for 10 minutes at 3,000 g at 2°C. Following the final centrifugation, the cells were resuspended in 1 ml sterile ice cold dH_2O. 1 to 5 µl of plasmid DNA was added to 200 µl of competent cells in an electroporation cuvette. The solution was electroporated in a BIORAD "Gene-Pulser" apparatus, with the voltage set at 2.5 kV and the Pulse controller set to 200 Ohms. Cuvettes were wiped to remove excess moisture and pulsed under the conditions described. 1 ml of SOC medium was added immediately following electroporation. The cells were transferred to a fresh micro-centrifuge tube and incubated at 37°C for 1 hour to express antibiotic resistance genes before being plated onto fresh LB plates containing the appropriate antibiotic. Remaining competent cells were stored in 10 % glycerol and frozen in aliquots at –80°C for up to 2 months. When required, the frozen aliquots were briefly warmed by hand for 1 – 2 minutes and placed on ice to thaw completely. Once thawed, the cells were used as described above.

Preparation of chromosomal DNA from bacterial cells.

- **The STEP method.**

The method used is as described by Silhavy *et al.*, (1984). 10 ml of the appropriate growth medium was inoculated with a single bacterial colony and grown overnight at the appropriate temperature. Cells were harvested by centrifugation at 8,000 g for 10 minutes in a Beckman J2-21 centrifuge (JA-14 rotor) and resuspended in 0.5 ml of 50mM Tris.HCl pH 8.0, 50mM EDTA (disodium salt) pH 8.0 in a 1.5 ml micro-centrifuge tube. The cell suspension was frozen at –20°C. Fresh lysozyme solution

(10mg ml^{-1} in 0.25M Tris.HCl pH 8.0) was added to the frozen cells which were thawed briefly with gentle mixing in a room temperature water bath. When the cell suspension had just thawed, they were placed on ice for 45 minutes, and 0.1 ml STEP solution was added. The solution was thoroughly mixed and heated at 50°C with occasional mixing. Nucleic acids were separated from the proteinaceous layer by extraction with an equal volume of $CHCl_3$ for 1 minute, followed by centrifugation at 15,000 g for 5 minutes in a bench top microfuge. The aqueous layer containing the nucleic acids was transferred to a fresh micro-centrifuge tube. The nucleic acid was precipitated by adding 0.1 x volume of 3M sodium acetate and 2 x volume of ethanol. The precipitate was spooled out using a glass micropipette and transferred to a clean tube containing 0.5 ml 50mM Tris.HCl pH 7.5, 1mM EDTA pH 8.0 and 200µg ml^{-1} RNaseA (70 K units mg^{-1} protein). The pellet was dissolved by rocking the tube gently at 4°C, overnight. An equal volume of $CHCl_3$ was added and mixed with the DNA solution by inversion. The aqueous layer containing the DNA was separated by centrifugation at 15,000 g for 5 minutes. The DNA layer was transferred to a fresh tube containing 0.1 x volume 3M sodium acetate and 2 X volume of ethanol. The DNA, which precipitated as long threads, was spooled out using a glass micropipette and dissolved completely in 0.4 ml of 50mM Tris.HCl pH 7.5, 1 mM EDTA, pH 8.0. The DNA was stored at –20°C. This procedure typically yielded 0.3 µg μl^{-1} DNA.

STEP solution contained 0.5 % SDS, 50mM Tris.HCl pH 7.5 and 0.4M EDTA (disodium salt). Proteinase K powder (12.9 unit mg^{-1} protein) (Sigma) was added to a final concentration of 1 mg ml^{-1} immediately before use.

- **The CTAB method.**

An alternative method of genomic DNA preparations from bacteria for Southern hybridisations was accomplished using the selective precipitation properties of hexadecyltrimethyl ammonium bromide (CTAB) (Murray and Thompson, 1980). Cell debris, polysaccharides and proteins can be removed from a bacterial lysate using CTAB and high-molecular weight DNA can be recovered by isopropanol precipitation.

The bacterial strain of interest was inoculated into 5 ml LB broth containing the appropriate antibiotic(s) and was grown overnight at 37°C with shaking. 1.5 ml of culture was transferred to a micro-centrifuge tube and cells were pelleted at 20, 000 *g* for 40 seconds. The supernatant was discarded and the cells were resuspended in 567 µl of TE buffer. 30 µl of a 10% (w/v) SDS solution and 3 µl of proteinase K (100 µg ml^{-1}) were added and the sample was mixed thoroughly. The sample was then incubated for 1 hour at 37°C with occasional mixing. 100 µl of 5 M NaCl was added and mixed in by inversion, prior to the addition of 80 µl of a CTAB/NaCl solution (10% [w/v] CTAB in 0.7 M NaCl). The sample was incubated at 65°C for 10 minutes and was then centrifuged at 20, 000 *g* for 5 minutes to spin out the CTAB-protein/polysaccharide complexes. The upper aqueous layer of viscous supernatant was then transferred to a new micro-centrifuge tube and an equal volume of phenol/chloroform was added and mixed in thoroughly. The tube was again centrifuged at 20, 000 *g* for 5 minutes and the upper aqueous layer was transferred to a fresh micro-centrifuge tube, 0.6 volumes of isopropanol was then added. The tube was gently inverted several times until a stringy white precipitate of the DNA appeared. The DNA was spooled out of the sample using a glass capillary tube and was transferred to a new micro-centrifuge tube containing 0.5 ml of 70% (v/v) ethanol, to remove the residual CTAB and NaCl. The DNA was then pelleted by centrifugation at 20, 000 *g* for 5 minutes, dried under vacuum and finally resuspended in 100 µl of TE.

Southern hybrisation and blotting.

Identification of specific sequences of DNA was established by the transfer technique of Southern (1975). In brief, DNA was separated by gel electrophoresis and transferred to a solid support *e.g.* nylon membrane. A radiolabelled DNA fragment was then hybridised to the DNA attached to the membrane and autoradiography was used to identify the position of the band complementary to the probe.

Genomic DNA of interest was prepared by the CTAB method and digested with restriction enzymes, the fragments were separated on the basis of size by agarose gel electrophoresis for 12-15 hours at 1 V cm^{-1}. The gel was photographed and the distance each molecular weight marker had migrated was recorded to allow size determination once the DNA had been transferred from the gel. The gel was soaked in 0.25 M HCl for 30 minutes, to depurinate the DNA, and then was rinsed in dH_2O to remove the excess HCl. Subsequently, the gel was soaked in 0.5 N NaOH, 1.5 M NaCl, for 40 minutes with gentle agitation on a moving platform, to denature the DNA. Finally, the gel was rinsed with dH_2O and treated with 0.5 M Tris. Cl, 1.5 M NaCl for a further 40 minutes with gentle agitation. Two strips of blotting paper were soaked in 20x SSC and placed on the upturned agarose gel casting tray, with the ends of the blotting paper, acting as wicks, resting in a reservoir of 20x SSC. The gel was turned upside down to ensure the DNA was closest to the surface of the gel and was transferred onto this support, ensuring no bubbles were trapped underneath the gel. One corner of the gel was removed to allow the orientation to be determined and all the sides of the gel were then overlaid with Clingfilm, to ensure the buffer could only move through the gel. Nylon membrane (Hybond^{TM}-N, Amersham) was cut to the size and shape of the gel and was laid directly on top of the gel, again ensuring no bubbles were trapped under the membrane. Three pieces of blotting paper were cut to the size of the gel, soaked in dH_2O and laid over the nylon membrane. A stack (6 cm) of paper towels which were also cut to the size of the gel, was then placed on top of the blotting paper producing a wick to draw liquid through the gel, thereby transferring the DNA to the nylon. Finally, a 500 g weight was placed on top of the stack to ensure good contact between the layers and the system was left overnight at room temperature. The following day the nylon membrane was retrieved and rinsed in dH_2O to remove excess SSC and then, was baked at 80°C for 2 hours which fixed the DNA to the membrane. Immediately prior to use, the nylon membrane was subjected to UV irradiation (1200 joules) for 2 minutes in a UV Stratalinker^{TM} 1800 (Stratagene) to cross-link the DNA to the membrane before hybridisation was performed with a radiolabelled DNA fragment.

High prime end-labelling.

Random prime labelling was performed as essentially described by Feinberg and Vodelstein (1983). Random hexanucleotides bind to the DNA fragment to be labelled and in the presence of nucleotides and the Klenow fragment of DNA polymerase I. The spaces in between the hexanucleotides were filled in using the parental strand as a template. One of the deoxynucleotides used was radiolabelled, hence the newly synthesised strand became radiolabelled.

DNA was labelled using the Boehringer Mannheim High Primed DNA labelling kit. Approximately, 50-100 ng of the DNA (0.2 kb upwards) to be labelled was made up to a volume of 11 µl with dH_2O in a micro-centrifuge tube and denatured by boiling for 10 minutes. The sample was immediately placed on ice, to maintain the DNA in a single-stranded form. 1 µl each of dATP, dTTP and dGTP were added to the single-stranded DNA in addition to 4 µl of Reaction buffer (containing the Klenow enzyme, hexanucleotides and buffer for the enzyme). 3 µl of [α-^{32}P]-dCTP (10 µCi μl^{-1}) was then added to make a final volume of 20 µl and the whole reaction mixture was incubated at 30°C for 15 – 30 minutes. The total volume was then made up to 200 µl and was separated through 1 ml of a TE-equilibrated pre-spun Sephadex G-50 column by centrifuging at 2, 000 *g* for 4 minutes. Unincorporated radio-isotope was retained in the column and the labelled DNA was eluted into a fresh micro-centrifuge tube.

Hybridisation and detection.

DNA hybridisations were performed at high-stringency (65°C) in a "Techne Hybridiser HB-1D" oven. A pre-hybridisation buffer containing 7% SDS (w/v), 0.5 M NaH_2PO_4 (pH 7.2) and 1 mM EDTA, and a hybridisation roller were incubated in the oven at 65°C. The nylon membrane, used to blot the DNA, was placed inside the roller and 20 ml of the pre-hybridisation solution containing 10 mg ml^{-1} denatured salmon testes DNA

was added to block the membrane. Meanwhile, the labelled DNA probe was denatured by boiling for 10 minutes. After 45 - 60 minutes the roller was removed from the oven and the original pre-hybridisation mixture was replaced with an equal volume of fresh buffer, along with the denatured probe. Hybridisation was allowed to proceed overnight after which the membrane was washed twice with a solution containing 5% SDS (w/v), 40 mM NaH_2PO_4 (pH 7.2) and 1 mM EDTA, followed by two washes of 1% SDS (w/v), 40 mM NaH_2PO_4 (pH 7.2) and 1 mM EDTA. All washes were performed at 65°C for 40 minutes and after completion the membrane was wrapped in Saran wrap and autoradiographed at -80°C.

DNA sequencing.

DNA sequencing was carried out using the ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Very pure plasmid DNA was prepared using a modified mini alkaline-lysis/PEG precipitation procedure.

E. coli cells harbouring the plasmid of interest were grown overnight in 5 ml of TB containing the appropriate antibiotic, at 37°C with shaking. Plasmid DNA was extracted using the small scale plasmid DNA extraction procedure described previously. Following ethanol precipitation, the plasmid DNA was resuspended in 32 µl of dH_2O and 8 µl of 4 M NaCl was added followed by 40 µl of autoclaved 13 % PEG_{8000}. The contents of the tube were mixed thoroughly and incubated on ice for 20 minutes to precipitate the DNA. The DNA was pelleted by centrifugation at 13, 000 *g* for 15 minutes, at 4°C. The supernatant was removed and the DNA pellet rinsed with 70 % ethanol. The pellet was dried in a vacuum for 5 minutes and resuspended in 20 µl of dH_2O. This method typically yielded 1.2 µg of plasmid DNA.

For the extension reaction, 3.2 pmol of the appropriate primer and 4 µl of reaction mixture were added to 0.5 µg plasmid DNA. (the reaction mixture, supplied as part of

the BigDye Termination Kit contained AmpliTaq DNA polymerase and the ddNTP terminators bonded to a fluorescein dye donor linked to a dRhodamine acceptor dye). The extension reaction was carried out as follows: 96°C for 30 seconds, 50°C for 20 seconds, 60°C for 4 minutes, for a total of 25 cycles. Following extension, the reaction products were precipitated by addition of 1 µl of 3 M sodium acetate, pH 4.6 and 25 µl of 95 % ethanol. The sample was mixed by vortexing and incubated at room temperature for 15 minutes, and covered in foil to exclude light, as the fluorescent dyes were light sensitive. The sample was then centrifuged at 13, 000 *g* for 20 minutes and the supernatant removed by pipetting. The DNA was rinsed with 250 µl of 70% ethanol and centrifuged for 5 minutes at 13, 000 *g*. The supernatant was removed and the DNA dried under vacuum for 3 minutes. The sample was stored at –20°C until it was loaded on the sequencing gel. DNA sequencing was carried out in an automated Perkin Elmer sequencer, by qualified personnel. The DNA sequence data was analysed using Gene Jockey software (Apple Macintosh).

2.2.3. β-galactosidase assays.

β-galactosidase assays were adapted from Miller (1972). Essentially, bacterial cells were lysed, which released the β-galactosidase. ONPG, which is colourless, was added which in the presence of β-galactosidase is converted to galactose and O-nitrophenol, which is yellow. The level of O-nitrophenol was measured, spectrometrically, which relates directly to the amount of β-galactosidase present in the cell.

Overnight cultures of the appropriate strains were diluted 1 : 100 into LB medium and grown, with shaking, at 37°C for 3 hours. The cultures were then split into 2 portions, 1 portion was placed in a water bath at 15°C and the other was placed in a water bath at 37°C. Samples were taken in triplicate every 30 minutes for 5 hours and the optical density at 600 mm was recorded. The samples were added to Z buffer, to a final volume of 1 ml. The cells were permeabilised by adding 100 µl CCl_3 and 50 µl of 0.1 % SDS

and vortexing for 30 seconds. 200 µl of 4 mg ml^{-1} ONPG (in 0.1 M phosphate buffer) was added. The time taken for a yellow colour to develop was recorded and the reaction stopped by addition of 500 µl of 0.5 M Na_2CO_3. The cells debris was removed by centrifugation at 13, 000 g for 30 seconds. 1 ml of the supernatant was transferred to a spectrophoretic cuvette and the optical density at 550 mm measured. The β-galactosidase activity was determined, in Miller units, using the following equation:

$$(O.D._{550} \times 1.75/ (t \times v \times O.D._{600})) \times 1000$$

where t is the time recorded for the yellow colour to develop and v is the volume of culture added to the Z buffer.

2.2.4. Rapid chilling assays.

Rapid chilling assays were adapted from Meynell (1938). An overnight culture was diluted 1:1000 in 100 ml NB, pre-warmed to 37°C. The culture was grown, with shaking, at 37°C for 5 hours. Parallel sets of 1 ml samples were removed from the culture every 30 minutes for 5 hours and diluted into 9 ml of 25 % Ringers solution which was either pre-warmed to 37°C or pre-chilled to 4°C. The samples diluted into the 25 % Ringers solution at 37°C were then serially diluted, as necessary, into 25 % Ringers solution and 200 µl aliquots were spread onto solid NB medium, in triplicate. The samples diluted into 25 % Ringers solution at 4°C were incubated at 4°C, in an electronically temperature controlled water bath, for 2 hours prior to plating on solid NB medium, in the same manner as for the samples at 37°C. The NB agar plates were incubated at 37°C for approximately 18 hours. Survival was calculated as a percentage of the CFU of the 4°C samples/ the CFU of the 37°C samples.

2.2.5. Bioluminescence assays.

Bioluminescence was measured from MPG361 (*cspB::*Mu*dlux*), MPG480 *cspB::*Mu*dlux rpoS*::*bla*), MPG481 (*cspB::*Mu*dlux*, *fis*::*cat*) at 30°C, 10°C and 4°C for

24, 72 and 96 hours respectively. The cultures were either in late exponential phase or in stationary phase of growth. For the exponential phase cultures, an overnight culture was diluted 2-fold into LB containing the appropriate antibiotic and incubated, aerobically, at 30°C for 1 hour. The culture was then divided 2-fold again and incubated for an hour, aerobically, at 30°C. The divisions and subsequent incubation periods were repeated for a total of 3 times in this manner. Immediately prior to measuring light production, the culture was divided 2-fold one more time and 200 µl samples pipetted, in triplicate, into white, 96 well microtitre plates. For the stationary phase samples, an overnight culture was diluted 100-fold into LB containing the appropriate antibiotic and grown for 24 hours, aerated at 30°C. The luminometer (Luminoskan RS) and the microtitre plates were equilibrated to the specified temperature for at least 1 hour before light production measurements were taken. As a negative control, bioluminescence from 3 wells with 200µl of LB was measured in addition to the strains mentioned previously. No bioluminescence was detected from the negative control wells during any of the experiments. As a positive control, bioluminescence from strain MPG361 was measured during each experiment. The bioluminescence detected from this strain was found to be reproducible for each of the temperatures tested, therefore, it was included as a control for the measurement of bioluminescence from other strains.

At the start of the light production measurements, the number of CFU was estimated by spreading some of the culture onto LB agar containing the appropriate antibiotic. The bioluminescent measurements are given as arbitrary RLU (relative light units) per bacterial cell.

2.2.6. 2 Dimensional polyacrylamide gel electrophoresis and protein detection and identification.

^{35}S – methionine/cysteine protein labelling.

For exponentially growing cells, an overnight culture of SL1344 was diluted 50-fold into 25ml of minimal Spitzizen media supplemented with histidine. The culture was grown aerobically at 37°C for approximately 3 hours, until the $O.D._{600}$ reached 0.5. Stationary phase cultures were similarly grown in minimal Spitzizen media for 24 hours. At these points 3ml was removed into 3 x 1.5 ml eppendorf tubes, 24 μCi of $^{35}S^{met/cys}$ (ICN) was added and the labelled sample incubated for a further 5 or 10 minutes at 37°C, for exponentially growing cultures or stationary phase cultures, respectively. The remainder of the culture was placed in a water bath at either 10°C or 4°C. Subsequent samples of 3 ml were removed at set time points and incubated with 24 μCi of $^{35}S^{met/cys}$ for 30 minutes or 60 minutes, for exponentially growing cultures or stationary phase cultures, respectively. The incubation times are described in table 2.8

	Temperature		
Growth phase	37°C	10°C	4°C
Exponential	5	30	30
Stationary	10	60	60

Table 2.8 Incubation times (minutes) for ^{35}S-methionine pulse labelled samples.

The labelled sample was centrifuged for 45 seconds at 12,000 x g to harvest the cells. The cells were resuspended in a total of 0.9 ml ice cold sonication buffer and placed on ice. The cells were then sonicated with 3 pulses each lasting 10 seconds. The lysed sample was then incubated on ice for 5 min with 50μl of 1 mg ml^{-1} DNAaseI. Urea was added to a final concentration of 9 M and an equal volume of 2D IPG sample buffer was added. The sample was loaded directly onto the prepared IEF strips or stored at -20°C.

Sonication buffer contained 0.01 M Tris.HCl, pH 7.4, 5 mM $MgCl_2$, 50 µg of pancreatic RNase ml^{-1} (Sigma). 2D IPG buffer contained 9M urea, 2% NP40, 2% IPG-buffer pH 3 – 10L (Pharamcia), 0.3% DTT, trace bromophenol blue.

IPG strips: reswelling and loading.

Pre-cast 11cm or 13cm IPG strips (Pharmacia) pH 3 –10 linear, or non-linear, were used in the 1st dimension. The strips were supplied dehydrated and required to be hydrated with the sample in 2D IPG buffer prior to running. 240 µl of sample was used to hydrate the 11cm IPG strips and 250 µl was used to hydrate the 13cm IPG strips. The sample in the 2D IPG buffer was placed in the appropriate slots in the Immobiline DryStrip Reswelling Tray (Pharmacia). The IPG strips were then placed on top of the sample. The IPG strips were then overlayed with mineral oil (Pharmacia) and allowed to hydrate for 10 – 18 hours.

1st Dimension gel electrophoresis.

The hydrated IPG strips were electrophoresed on a Multiphor II apparatus (Pharmacia). The IPG strips were placed in an alignment tray immersed in mineral oil. Dampened wicks and electrodes were placed over the basic (cathode) ends of the IPG strips the acidic (anode) end of the IPG strips. The 11cm IPG strips were pre-run at 300V for 1 Vhr and run at 14,000V for 20 Vhr. The 13cm IPG strips were pre-run at 300V for 1 Vhr and run at 20,000V for 17 Vhr. After electrophoresis, the IPG strips were either stored at –80°C or immediately equilibrated in 2D equilibration buffer and transferred to cast slab gels for 2nd dimension electrophoresis.

Equilibration of the IPG strips.

The IPG strips were placed into 25 ml glass tubes containing 15ml of equilibration buffer A (IPG strips that had been stored at –80°C were allowed to thaw for 2 or 3 minutes). The tubes were sealed and placed on their side on a rocking tray and gently agitated for 10 minutes at room temperature. The IPG strips were then transferred into 25ml test-tubes containing 15ml of equilibration buffer B. The tubes were again sealed and agitated gently for 10 minutes at room temperature. 2D Equilibration buffer contained 50 mM Tris.HCl pH 6.8, 4 M urea, 12% glycerol and 4% SDS. Equilibration buffer A contained 25 mg DTT per 10 ml of equilibration solution. Equilibration buffer B contained 0.45 g iodoacetamide and trace bromophenol blue per 10 ml of equilibration solution.

SDS polyacrylamide slab gel preparation.

The polyacrylamide slab gels for the 2nd dimension were cast in an SE 600 gel electrophoresis apparatus (Hoefer Scientific Instruments). The dimensions of the gel were 15 cm x 15 cm and either 1 mm or 1.5 mm, depending on the procedure. The 16.5% T 3% C resolving gel solution was poured to a height of 10cm, a 10% T 3% C spacer gel solution of 3 cm was overlaid on the resolving gel and a 4% T 3% C stacking gel solution of 1.5 cm completed the slab gel. The composition of the gel solutions is shown below.

	Stacking gel solution	Spacer gel solution	Resolving gel solution
Acrylamide solution[1]	1.0 ml	6.1 ml	10.0 ml
Gel Buffer[2]	3.1 ml	10.0 ml	10.0 ml
Glycerol	-	-	3.2 ml
Water	8.4 ml	13.9 ml	6.8 ml
APS[3] (10 % (w/v))	0.1 ml	0.1 ml	0.1 ml
TEMED[4]	0.01 ml	0.01 ml	0.01 ml

Table 2.9 Solutions for SDS-PAGE.

1: acrylamide solution contained 48 % (w/v) acrylamide and 1.5 % (w/v) N,N'-methylene-bis-acrylamide. 2: gel buffer contained 3 M Trizma base and 0.3 % (w/v) SDS, adjusted to pH 8.45 with HCl. 3: APS - Ammonium persulphate. 4: TEMED - N,N,N'-tetramethylethylethylenediamine.

2nd dimension gel electrophoresis.

Following equilibration, the IPG strips were transferred to the prepared SDS-PAGE slab gels for 2nd dimension electrophoresis. Tricine buffered SDS-PAGE was performed as described by Schagger and VonJagow (1987). The procedure dissociates proteins into their individual sub-units and subsequently separates them according to their size. Tricine was used as an alternative buffereing solution to separate proteins between 2.5 and 70 KDa. Glycine is commonly used to separate proteins over a higher range of molecular weight, e.g. 10 – 90 KDa. However, since tricine migrates faster than glycine (as more tricine is in the migrating, anionic form), proteins with a smaller molecular weight are more easily resolved.

The IPG strips were drained for 2 or 3 minutes on Whatman 3M filter paper to remove excess equilibration buffer and transferred to the top of the polyacrylamide slab gel. 10µl of tricine molecular weight markers (Sigma) were pipetted onto a small piece of filter paper and placed beside the IPG strip. The IPG strip and molecular weight markers were sealed on to the top of the slab gel using 1 % w/v molten agarose, which was not more than 50°C. Once the agarose had solidified, cathode buffer was poured into the upper buffer chamber. The lower buffer chamber was filled with anode buffer. The

electrophoresis was carried out at 30 mA per gel for 16 hours at 4°C, or until the tracking dye was 1 cm from the base of the gel. Once electrophoresis was completed the gel was removed and either stained with coomassie blue or the proteins were blotted onto PVDF membrane for sequencing or nitrocellulose membrane for immunodetection.

Coomassie Blue Staining

The proteins were fixed in the gel by soaking the gel in 50 % methanol, 10 % acetic acid for 2 hours, with gentle shaking. The gel was then stained with 1 % (w/v) coomassie blue G (Sigma) in 10 % acetic acid until the protein spots were visible. The gel was destained in several changes of 10 % acetic acid until the protein spots were visible against a clear background.

Gel drying and autoradiography

The gels were dried by first soaking in a solution of 35 % ethanol, 5 % glycerol for 2 hours. The gel was placed on 2 pieces of Whatman 3M filter paper, overlaid with Saran wrap and dried on a vacuum gel drier. Once the gels were dried, the Saran wrap was removed and the gels placed in an autoradiography cassette with X-ray film (Kodak). The X-ray film was developed in an automatic developer and the autoradiographs were scanned into the computer. As the autoradiographic exposure time was the same for all the gels from each experiment, differential protein synthesis could be quantified. It should be noted that the labelling time at 37°C was only 16 % that of the cold shocked samples, therefore the radioactive signal was a 6-fold underdisplay relative to the gels of the cold shocked samples. The autoradiographs were scanned into a computer and protein synthesis from the 2-D PAGE gels was analysed using Phoretix 2-D software (v 4.0).

2.2.7. Electro-blotting proteins and amino terminal protein sequencing (Edman degredation method).

Following molecular weight separation, proteins were blotted onto PVDF Immobilon-P membrane (Millipore) using a Trans Blot Cell (BIORAD). The proteins were then excised from the membrane and sequenced by Edman degredation.

Subsequent to SDS-PAGE, the slab gels were soaked in CAPS transfer buffer with gentle shaking, for 15 minutes. The PVDF membrane pieces, 15 cm x 15 cm, were wetted in methanol Prior to soaking in CAPS transfer buffer for 5 minutes,. A gel/membrane sandwich was assembled, with each component pre-soaked in CAPS transfer buffer. The membrane was placed on top of the gel which was sandwiched between 4 pieces of Whatman 3M filter paper, also 15 cm x 15 cm, and 2 Scotbrite pads. The sandwich was placed in a cassette and immersed in CAPS transfer buffer in the electro-blotting tank (Biorad). Electroblotting was carried out at 20V for 16 - 24 hours at 4°C. The PVDF membrane was removed and washed in several changes of d.H_2O and stained with 1 % (w/v) amido black in 50 % methanol for 15 minutes, with gentle shaking. The membrane was destained with several changes of a destain solution containing 50 % (v/v) methanol and 10 % (v/v) acetic acid until the protein spots were visible against a clear background. The PVDF membrane was dried by soaking in Methanol for 10 seconds and allowing the dry in air for 15 minutes. The protein spots of interest were cut out using a sharp scalpel blade and stored at –20°C.
CAPS transfer buffer contained 10mM CAPS (3-[cyclohexylamino]-1 propane sulphonic acid) pH 11, and 10 % methanol (v/v).

N-terminal amino acids were determined with automated sequencing on an Applied Biosystems 477A instrument according to the method describe by Hayes *et al.*, (1989)

35-S methionine protein labelling enhancement

This procedure was only used to enhance the signal from particularly low intensity protein spots. Following radio-active labelling with ^{35}S-met/cys and subsequent 2D electrophoresis, proteins were electroblotted onto PVDF membrane. The membrane was embedded with scintillantion wax by sandwiching the membrane and wax pieces with 2 warmed glass plates. An even pressure was applied by clamping the glass plates with bulldog clips at each side. Once the wax had spread throughout the membrane, it was cooled briefly to set the wax. The embedded membrane was exposed to X-ray film for 24 hours before being developed.

CHAPTER 3

MUTATION OF *S. typhimurium cspA*

3.1 INTRODUCTION

A principal feature of the bacterial cold shock response is induction of a novel group of highly related proteins, of which the major protein is normally termed CspA. In most bacteria, there are several members of the CspA family. For example, there are at least 4 parologous proteins in *S. typhimurium* (see Chapter 6 of this thesis) and 9 in *E. coli* (for a recent review see Yamanaka, *et al.*, 1998). Indeed, it has been postulated that the proteins have arisen through gene duplication events, and then diversified both at the level of gene structure and regulation, and also at the level of protein structure and function. In *E. coli*, 6 of the genes are clustered on the chromosome in 2 similar segments, each containing 3 genes, but with separate regulatory features (Yamanaka *et al.*, 1998). Moreover in some bacteria, only some of the CspA-like proteins are cold induced. For example, both CspA and CspB of *S. typhimurium* (Chapter 6 of this thesis; Craig *et al.*, 1998) are cold induced as are *E. coli*, CspA, CspB, CspG and CspI (Goldestein *et al* 1990, Lee *et al.*, 1994, Nakashima *et al.*, 1996, Wang *et al.*, 1999). CspA-like proteins that are cold induced are termed CSPs (cold shock proteins) and consequently the genes are collectively termed *csp* genes.

3.1.1 Survival of bacteria at low temperatures.

In order to survive and grow at sub-optimal temperatures, bacteria must adapt in several ways. Reduction of ambient temperature leads to a decrease in membrane fluidity, which must be overcome to restore membrane function. In addition, translation initiation is blocked and in *E. coli*, an accumulation of 70S ribosome sub-units has been reported, following a shift from 37°C to 5°C (Broeze *et al.*, 1978). When a culture of *E. coli* was shifted to 10°C, a temperature which is permissible for growth, there was an initial lag in growth, during which time the majority of *de novo* protein synthesis was blocked. At the same time, specific proteins, termed cold inducible proteins, were highly induced, and these included CspA. However after several hours, the block in translation of the majority of proteins was overcome (Jones *et al.*, 1987). Some proteins, termed cold acclimation proteins, were found to

be synthesised at higher levels at 10°C than at 37°C, after the block in translation was overcome. CspA (and related cold induced family members) are thought to play a major role in adaptation to the sub-optimal temperatures.

CspA homologues have been found in a wide range of bacterial species, and increasing evidence supports their role in adaptation to survival in the cold (for recent reviews see Yamanaka *et al.*, 1998; Panoff *et al.*, 1998). For example, cold inducible CspA homologues have been identified in nine commercially important strains of lactic acid bacteria. Interestingly, when exponentially growing cultures of *Lactococcus lactis* were shifted from 30°C to 10°C there was no lag in growth, unlike that observed in *E. coli* following a shift from 37°C to 15°C, which suggests that this species is better able to grow at low temperatures than *E. coli*. In addition, cell survival after repeated freeze-thawing was found to significantly improve if the cultures were cold shocked to 10°C prior to freezing. For example, survival of 3 strains of *L. lactis* increased by approximately 30% when the cultures were shifted to 10°C for 2 hours prior to freezing and survival increased further, approximately 50%, when the pre-freeze incubation at 10°C was increased to 5 hours (Kim & Dunn, 1997).

Studies in *B. subtilis* were the first to show that CspA homologues were necessary for survival at low temperatures. *B. subtilis*, like *E. coli* and *S. typhimurium*, is a mesophile, and has a similar minimum temperature at which growth is permissible to that of *E. coli*. There are 3 known CspA homologues in *B. subtilis*, the major cold shock protein, CspB and 2 additional cold inducible proteins, CspC and CspD (Graumann *et al.*, 1997). Deletion of any two of the *cspA* homologous genes led to a severe restriction in growth and division at 15°C, although no effect on survival was observed when only a single homologue was disrupted and the mutant was grown at either 37°C or 15°C. Moreover, disruption of all three *csp* genes simultaneously, proved lethal at optimal temperatures. Indeed, complementation, where *cspB* was placed under the control of the ITPG-inducible *spac* promoter on a plasmid, was required to allow construction and isolation of the triple mutant. The plasmid was

also found to be maintained in the triple mutant even in the absence of antibiotics (Graumann *et al.*, 1997).

However, following freezing to –80°C, survival of the *B. subtilis cspB* null mutant (*cspB::cat*) was severely compromised. The percentage of viable cells decreased from approximately 27% for the parental strain to 2% for the *cspB* mutant (Willimsky *et al.*, 1992). In addition, a difference in protein synthesis in the cold was observed. CspB appeared to affect the induction of several cold inducible proteins (CIPs) at 15°C, so that induction of 15 of these proteins increased in the *cspB::cat* strain, while the expression of 7 other CIPs remained at the same level as was observed at 37°C. Yet, these differences did not affect the ability of this mutant to grow as well as the wild type cells, following a shift from 37°C to 15°C (Graumann *et al.*, 1996).

Deletion of *cspA* in *E. coli* did not lead to a decrease in survival following a temperature decrease from 37°C to 15°C, relative to the parental strain. However, 2-D analysis showed that following the same temperature decrease, the production of CspB and CspG increased slightly, by approximately 1.3 fold. In addition, their expression was prolonged relative to production in the wild type strain, for at least 3 hours (Bae *et al.*, 1997). These results are consistent with the changes observed in the level of induction of cold inducible CspA homologues in *B. subtilis*, following deletion of some of the cold shock proteins (Graumann *et al.*, 1997). Simultaneous deletion of any 2 CspA-like proteins resulted in increased synthesis of the remaining functional CspA-like protein, at 37°C. Furthermore, following a temperature decrease from 37°C to 15°C, CspC was found to be induced to a very high level, relative to the control culture, when CspB and CspD were mutated. Thus, it appears that in an *E. coli cspA::cat* disruption strain, and in *B. subtilis* strains that are mutated for 2 CspA-like proteins, other proteins and perhaps CspA homologues, may compensate for the absence of the major cold shock protein. Interestingly though, in *E. coli* the levels of induction of other cold induced CspA homologues do not reach those achieved by CspA, even in a strain in which CspA has been disrupted.

3.1.2 Function of CspA homologues.

Although there is a reasonable body of work on *E. coli* CspA, its exact role in the cold shock response has still to be fully elucidated *in vivo*. CspA shares similarity with a family of proteins found in eukaryotic cells, called Y box proteins. Many CspA homologues, including *S. typhimurium* CspA and CspB, *E. coli* CspA and *B. subtilis* CspB, contain 2 RNA binding sequences, similar to those found in Y-box proteins (Didier *et al.*, 1988). The RNP1 sequence is conserved and the second RNP2 sequence is partially conserved to the consensus sequence (figures 1.2 and 1.3 of this thesis) (Schindelin *et al.*, 1994; Graumann *et al.*, 1994; Craig *et al.*, 1998). Some eukaryotic Y-box proteins such as DBP-1 have been shown to act as transcriptional regulators (for a review, see Wolffe *et al.*, 1993) and studies of *E. coli* CspA and *B. subtilis* CspB and CspD have demonstrated that these proteins are capable of binding to the nucleotide sequence ATTGG, the highly conserved core of the Y-box sequence. In studies of *B. subtilis* CspB, which utilised single or double stranded nucleic acid 54-mers containing either the ATTGG or CCAAT motifs, CspB was found to bind with highest affinity to ssDNA containing ATTGG. Complete retardation of 15 pmols of the 54-mer was observed in gel shift assays with 30 pmols of CspB. The binding affinity to the 54-mer containing the complementary sequence was reduced by 50 %. However, CspB did not appear to bind the dsDNA version of the 54-mer (Graumann *et al.*, 1994). Further studies showed that *B. subtilis* CspB and CspD also bound RNA (Graumann *et al.*, 1997). Complete retardation of 25 pmol of RNA occurred with 200 pmol of CspB and 400 pmol of CspD. The affinity of CspB for the corresponding ssDNA was approximately 2-fold higher, so that 125 pmol CspB protein retarded 25 pmol of ssDNA. In this case, the 24-mer DNA, (from which the RNA was derived), was a truncated version of the 54-mer that still contained the ATTGG (Graumann *et al.*, 1997). Interestingly, although *B. subtilis* CspC has more than 70% identity to CspB, the binding affinity of CspC (which is cold inducible) for a range of ssDNA molecules was markedly lower than the binding affinities of CspB (Graumann *et al.*, 1997).

Gel shift assays have shown that purified *E. coli* CspA, in 2–10-fold molar excess, bound duplex DNA derived from the *gyrA* promoter. This region of DNA contains 3 ATTGG sequences, at positions –112, -92 and –63 with respect to the transcriptional start site, and addition of purified CspA resulted in formation of 3 DNA/protein complexes (Jones *et al.*, 1992). Mutation of 2 of the sequences resulted in only 1 protein-DNA complex. Mutation of all 3 sites eliminated protein-DNA binding completely (Jones *et al.*, 1992). *E. coli* CspA, from cold shocked crude cell extracts, was also shown to bind to duplex DNA derived from the promoter region of *hns* (Brandi *et al.*, 1994). Although the *hns* gene of *E. coli* and *S. typhimurium* contains the CCAAT sequence close to the promoter (upstream of the transcriptional start site), the equivalent region in *Proteus vulgaris* lacks this sequence. Yet reporter studies showed that *P. vulgaris hns* was cold shock inducible (Brandi *et al.*, 1994). Thus, it appears that the CCAAT sequence is not stringently required for CspA binding. Additional factors that were present in the crude cell extracts also appeared to be involved in binding, since the affinity of purified CspA for *hns* DNA was substantially lower than that of CspA from the crude cell extract. An equivalent degree of retardation of the protein/DNA complex was formed by the addition of 16.2 μM of purified CspA compared to 6 μg of total protein extract, with duplex DNA derived from the promoter region of *hns*. Furthermore, purified CspA has been shown, *in vitro*, to enhance the expression of *hns* from a plasmid based *hns-cat* fusion (Brandi *et al.*, 1994). This evidence, together with preferential binding of *E. coli* CspA and *B. subtilis* CspB to core Y-box sequences, indicates that these proteins may act as transcriptional activators.

More recently, it has been shown that *E. coli* CspA also interacts with ssRNA and may act as an RNA chaperone (Jiang *et al.*, 1997). Purified *E. coli* CspA was added to a mRNA 159-mer oligonucleotide, which contained the 5' untranslated region (5'-UTR) of *E. coli cspA*, and resulted in enhanced degradation of the mRNA in the presence of RNaseA and RNase T1. However, the amount of purified CspA that led to a retarded protein/RNA complex was 2.7×10^{-5} M with gel electrophoresis, and the authors have suggested that since the amount of CspA required for the shift was relatively high, the binding may have been co-operative. (Jiang *et al.*, 1997). These

results led to the proposal that CspA is an RNA chaperone, preventing RNA mis-folding and resolving mis-folded RNAs at low temperature, thereby maintaining translational capacity. It is noteworthy that the RNA substrate used in these assays was the 5-UTR of *cspA* mRNA, therefore the results showed that CspA may act to chaperone its own RNA (Jiang *et al.*, 1997).

3.1.3 Expression of CspA homologues.

Several β-galactosidase fusions to CspA homologues have been constructed at the genetic level to explore both transcriptional and translational expression in the cold. In one study, *E. coli* cells carrying *cspA-lacZ* fusions integrated on the chromosome exhibited a 2.5 to 3-fold increase in β-galactosidase activity following a shift from 37°C to 15°C for 4 hours, and the results were similar for both transcriptional and translational fusions. In this case, the *lacZ* gene was fused at position +81 bp or +346 bp from the transcriptional start site of *cspA*, respectively, (Goldenberg *et al.*, 1996). In contrast, a different study found that *lacZ* fusions to the 10^{th} and 11^{th} codon (at positions +190 and +193, respectively) of *E. coli cspA* and *cspB* respectively, showed approximately 16-fold increases in β-galactosidase activity after incubation at 15°C for 4 hours. In the latter study however, the *lacZ* fusions were plasmid based rather than chromosomally based, although a *pcnB* mutant was used as the host strain in order to keep the copy number a low as possible (Lee *et al.*, 1994). The *pcnB* mutation is known to maintain pBR322 plasmids and its derivatives at approximately 2 - 3 copies per host cell (Lopilato *et al.*, 1986).

A *B. subtilis cspB–lacZ* chromosomal fusion exhibited approximately a 6 to 8 fold increase in β-galactosidase activity following a 60 minute incubation at 10°C (Willimsky *et al.*, 1992). In this study the *lacZ* gene was inserted immediately downstream of the ribosome binding site of *cspB*, (at position +112 in relation to the transcriptional start site, the translational start site is located at base +119) (Willimsky *et al.*, 1992). It is worth noting that when a similar *cspB-lacZ* fusion that contained 474 bp of the *cspB* promoter region, was integrated onto the chromosome

in a *B. subtilis* SPβ prophage attachment site, no β-galactosidase activity was detected from this fusion following a temperature reduction. This led to the suggestion that a *cis*-acting element, which played an essential role in cold shock regulation, lay upstream of this region of *cspB* and was lacking in the chromosomally disrupted *cspB-lacZ* fusion strain. To address this issue, a strain was constructed which contained the wild type copy of *cspB* as well as the *cspB-lacZ* fusion. In this case the mutated version of *cspB* was integrated into the chromosome by a single cross-over event, upstream of the wild type copy of *cspB*, which resulted in cold shock induction of *cspB* expression from the *cspB-lacZ* fusion. In this way, *cspB-lacZ* activity was monitored in the presence of the putative *cis* acting element (Willimsky *et al.*, 1992).

3.1.4 Regulation of genes encoding CspA homologues.

Much of the molecular basis of the regulation of the cold shock genes has been deduced in *E. coli*. (This topic will be more extensively described in Chapter 4 of this thesis, however, the following provides a brief overview of some of the major findings). In particular, regulation of the major cold shock gene, *cspA* has been well studied. The major factor in regulation of cold shock genes appears to be stability of the mRNA transcript (Goldenberg *et al.*, 1996). For example, the half life of the *E. coli cspA* mRNA transcript is approximately 15 seconds at 37°C, whereas prolonged incubation at 15°C resulted in an increase to approximately 60 minutes (Goldenberg, *et al.*, 1996). In this case, the *E. coli* cultures were in exponential phase. There appears to be differences in the thermoregulation of various members of the CspA family. For example, studies with *S. typhimurium cspB* have shown that the mRNA transcript is stabilised below a threshold temperature of approximately 22°C (Craig *et al.*, 1998). These findings were substantiated with reporter *cspB* translation fusion studies. Similarly, *E. coli cspB* seems to be stabilised below a threshold temperature of around 20°C, and CspB protein induction studies showed similar results (Etchegaray *et al.*, 1996). In contrast, it appears to be the degree of temperature shift which induces *cspA* expression. (Etchegaray *et al.*, 1996). Studies with *E. coli cspA*

have indicated that RNaseE may play a major role in the degradation of the cold shock genes at 37°C (Fang *et al.*, 1997).

Other factors also appear to play a role at the level of transcription and translation. The 5'untranslated region of the cold shock genes is particularly long and contains elements, other than the promoter, that affect expression of the genes. For example, a putative Up element, an AT-rich region upstream of the promoter region, appears to enhance transcription of *E. coli cspA* (Mitta *et al.*, 1997). Deletion of this region in *E. coli cspA* resulted in a 4-fold decrease in expression, following incubation at 15°C for 3 hours, as measured from β-galactosidase levels from a *cspA-lacZ* transcriptional fusion (Mitta *et al.*, 1997).

In addition, a region present in the initial 10 codons of the translational sequence of *E. coli cspA* also appears to enhance transcription of *cspA* (Mitta *et al.*, 1997). This has been termed the downstream box and expression of *E. coli cspA* was shown to be deleteriously affected when this region was removed, as measured from *cspA-lacZ* fusion constructs (Mitta *et al.*, 1997).

In contrast, an element termed the cold box, which is present in *E. coli cspA, cspB* and *csdA* was shown to be involved in the transient expression of these genes. Deletion of this region of DNA resulted in prolonged expression of both the genes and the gene products (Fang *et al.*, 1998). Thus it seems that there is a degree of autoregulation of the cold shock genes.

3.1.5 Aims of this chapter.

In light of the emerging information on regulation of the cold shock response in *B. subtilis*, *E. coli* and other bacteria, it seemed important to disrupt the chromosomal copy of *S. typhimurium cspA*. This would provide insight into the regulation of *cspA* and indicate whether regulatory differences exist between *E. coli* and *S. typhimurium* in regulation of the cold shock response. This chapter describes attempts to disrupt

the cloned *S. typhimurium cspA* locus by insertional inactivation, initially using the *lacZ* reporter gene and subsequently a *cat* cassette, followed by allelic exchange onto the chromosome.

3.2 RESULTS

The *S. typhimurium cspA* locus had been previously identified and cloned in our laboratory (J. Craig & M.P. Gallagher, unpublished data) (figure 3.1). In order to achieve this, the location of *cspA* paralogues in *S. typhimurium* were mapped by dot blot and Southern blot analysis using lysates from the Mu*d*P22 mapping kit (Benson & Goldman, 1992) and the *E. coli cspA* as a probe. This revealed that a paralogue existed at approximately 79 genetic minutes, within an *EcoR*I fragment of approximately 4 kb. This *EcoR*I fragment was subcloned into the corresponding site in the vector pBluescript KS. Subsequent DNA sequencing showed that the *cspA* ORF was present near one end of the fragment and was preceded by approximately 400 bp of upstream sequence, as shown in figure 3.2. The *EcoR*I fragment also contained approximately 3.5 kb of unknown sequence downstream of the *cspA* ORF. The resulting plasmid was termed pJEC17 (figure 3.2).

3.2.1 Deletion of the *S. typhimurium cspA* ORF from pJEC17.

In order to disrupt the *cspA* locus by removing the ORF, a series of intermediate clones were made. Initially, the ORF and all of the 5' region of *cspA* were removed from pJEC17 using a native *Hind*III site at the 3' end of the ORF of *cspA* and the *Hind*III site present in pBluescript KS (figure 3.2), which resulted in the construction of pLJF1 (figure 3.3 A). Subsequently, the region, from position – 265 to + 110 which lacked the ORF, was re-introduced at the start of the truncated *cspA* gene. The 5' untranslated region (UTR), including the promoter of *cspA*, was amplified by PCR from pJEC17, using primers MICG1 and MICG2 (table 2.3, chapter 2 of this thesis). MICG1 contained bases 1 to 23 (position –265 to –242, with respect to the transcriptional start site) and incorporated artificial *Sal*I and *Xba*I restriction sites at the 5' end. MIGC2 was complementary to bases 376 – 336 (position + 111 to + 71, with respect to the transcriptional start site) (on the non-coding strand) and incorporated artificial *Bgl*II and *Hind*III restriction sites at the 5' end.

```
1     GCCAGAGCGC TGGGCGTAAC GGTTGCCATG GTTCAGGATG GGATCGAAAC

51    GCGAAAACCC ACGCCAGCAG AGCTTAAATT AATGCGCCTG ATACAGGCGA
                         Y-box
101   CCCACGCTTA AGTAAGCaat tgATGGAGTA ATTTTTACCC CTTTCTGTTT

151   TTAACGGTCC TGCGTAAGGA CCGTTTTCCC CGCCCGATTA CTGGCCTGGA
            Up element          -35                    -10
201   GAAATAAAGT aaaataaaag TTGCATCGCC CGCCATTACA TGAGTTAATG
                    (+1) Cold box
251   TGCTCAACGG TTTgAcgtac agaCCATTAA AGCAGTTTAG TAAGGCAAGT

301   CCCTTCAAGA GTTATCCATT AGATACCCCT CGTAGTGCGC ATTTCCTTAA

351   CGCTTAAAAA ATCTGTAAAG CACGCCATAA CGCCGAAAGG CACGACGTTA
          RBS
401   TTTTTTAAAA GGTAATACAC TATGTCCGGT AAAATGACTG GTATCGTAAA
                                M  S  G    K  M  T    G  I  V  K

451   ATGGTTCAAC GCTGATAAAG GCTTCGGCTT TATTACTCCT GATGACGGTT
      W  F  N  A  D  K  G  F  G  F    I  T  P  D  D  G  S
                                RNP1
501   CTAAAGACGT GTTCGTACAC TTCTCCGCTA TTCAGAACGA TGGTTACAAA
        K  D  V  F  V  H  F  S  A  I  Q  N  D  G  Y  K
                   RNP2

551   TCTCTGGACG AAGGTCAGAA AGTTTCCTTC ACCATCGAAA GCGGCGCTAA
      S  L  D  E  G  Q  K  V  S  F  T  I  E  S  G  A  K

601   AGGCCCGGCA GCTGGCAACG TAACCAGCCT GTaagcttAA AAGCTCAGCA
        G  P  A  A  A  N  V  T  S  L  *

651   TTTTGATCCT GCTGATGGCG
```

Figure 3.1 DNA sequence of *S. typhimurium cspA*.

The nucleic acid and derived amino acid sequence of *S. typhimurium cspA* (J. Craig, unpublished). The ribosome binding site, -35 and -10 sites are underlined, the transcriptional start site is in italics. The putative Y-box, UP element and cold box regions are shown in lower case. The *Hind*III restriction enzyme site located at the translation stop codon is also shown in lower case. In the amino acid sequence, the RNP1 and partial RNP2 sequences have been underlined.

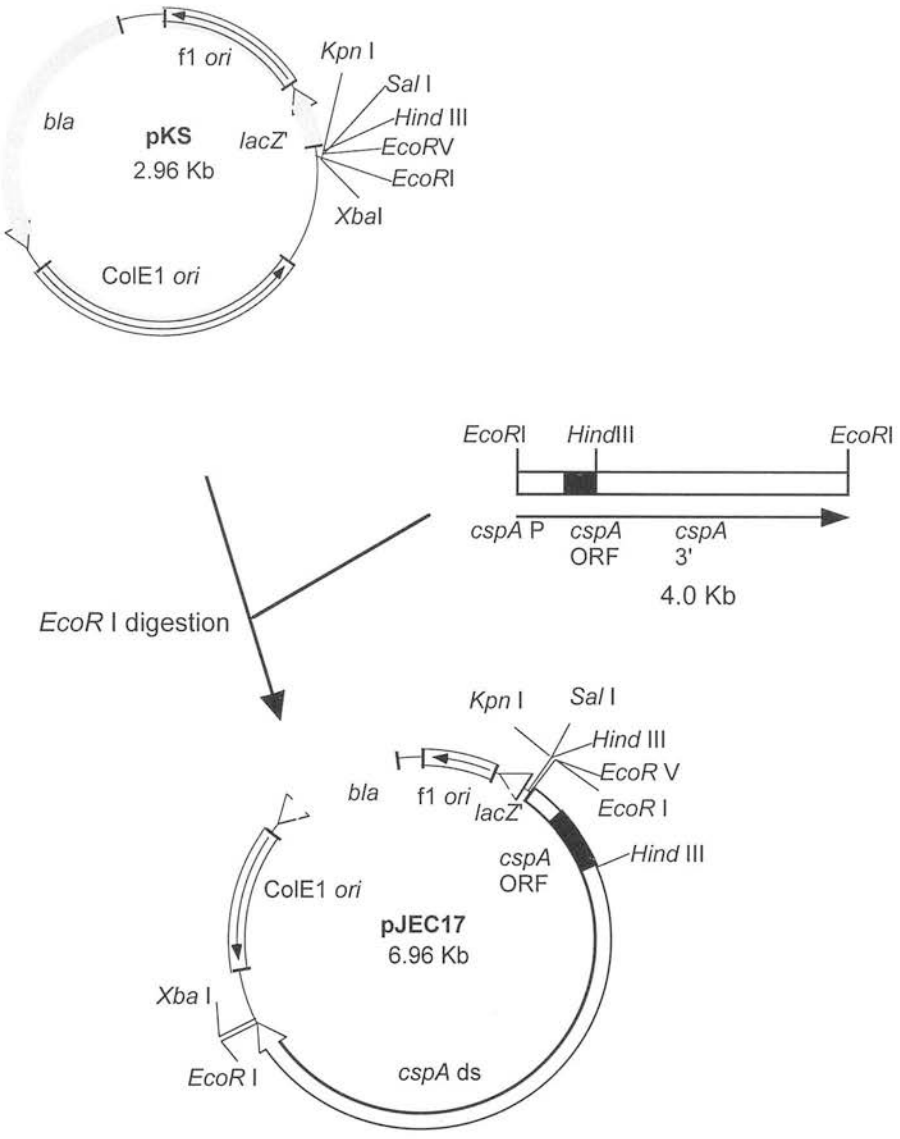


Figure 3.2 Construction of pJEC17.

A 4.0 Kb fragment containing *S. typhimurium cspA* (located approximately 265 bp upstream of the transcriptional start site of *cspA* to approximately 3.5 kb downstream of the *cspA* stop codon) was cloned into the *EcoR* I site in the pKS vector, forming pJEC17 (J. Craig, unpublished).

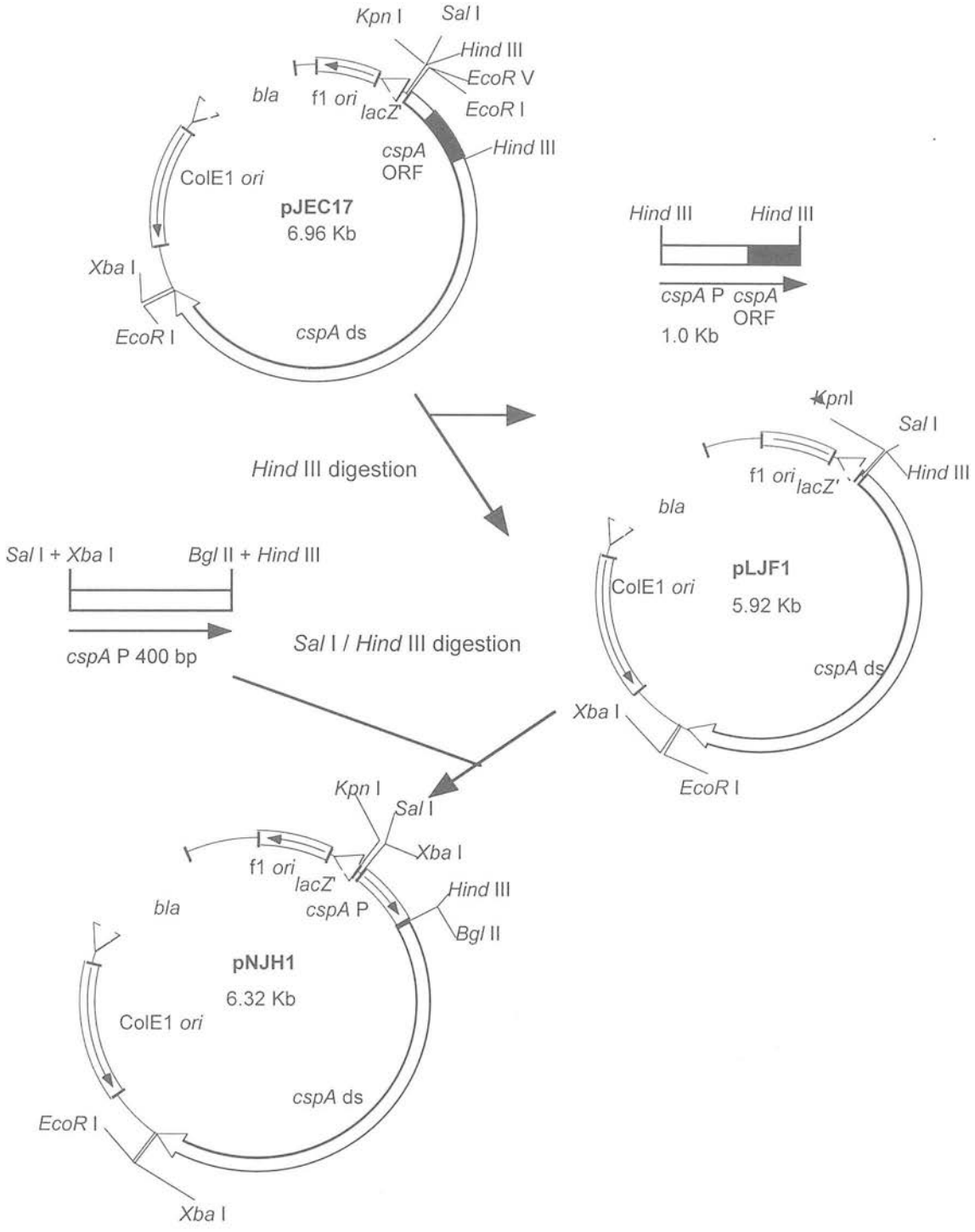


Figure 3.3 A Construcion of pLJF1 and pNJH1.
The promoter region and ORF of *cspA* (from bases -254 to +368) was deleted from pJEC17 by digestion with *Hind* III, forming pLJF1 (L.J. Foster, unpublished). The promoter region of *cspA* was re-introduced by PCR amplification of theis region (from bases -264 to +113) from pJEC17, using primers MICG1 and MICG2. *Sal* I and *Xba* I sites were incorporated into the 5' primer and *Bgl* II and *Hind* III sites into the 3' primer. The DNA was cloned into the compatible sites in pLJF1, forming pNJH1.

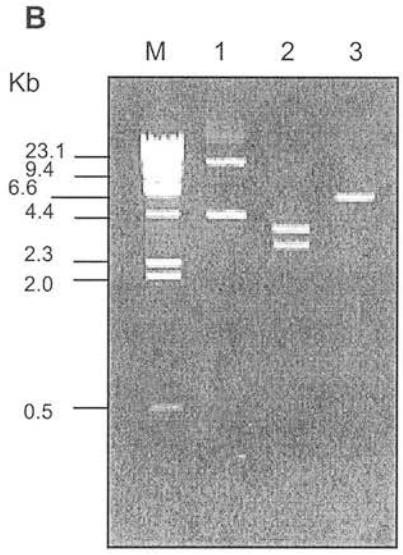


Figure 3.3 B: Restriction analysis of pNJH1.

Lane 1, pNJH1 un-digested; lane 2, pNJH1 digested with *Xba*I; lane 3, pNJH1 digested with *Hind*III and *Bgl*II; M, DNA ladder (λ DNA digested with *Hind*III), the sizes and positions have been indicated.

The PCR amplified DNA was cloned into the *Sal*I and *Hind*III restriction sites of pLJF1 and the resulting plasmid was termed pNJH1 (figure 3.3, panel A). This plasmid contained 376 bp of DNA upstream of the start of the *cspA* ORF and approximately 3.5 Kb of DNA downstream from the translational stop codon of the *cspA* ORF. For the purpose of this thesis, these regions of DNA are termed *cspA* P and *cspA* ds, respectively. Correct construction of pNJH1 was confirmed by restriction analysis of the DNA (figure 3.3 B). Restriction of pNJH1 with *Xba*I produced a 4.0 Kb fragment corresponding to the *cspA* P and *cspA* ds region, and another fragment which corresponded to the remainder of the pKS vector. Co-digestion with *Bgl*II and *Hind*III generated a single DNA fragment of approximately 6.5 Kb which corresponded to the whole pNJH1 plasmid.

3.2.2 Replacement of the *cspA* ORF with *lacZ-cat* within pNJH1.

Once the *cspA* ORF was removed from pJEC17, the *lacZ* gene and the *cat* cassette were inserted sequentially. This would allow the activity of the *cspA* promoter to be monitored via β-galactosidase expression. The *cat* cassette provided a means of identifying chromosomal integrants following recombination with the chromosome. The first step of this process was to subclone the *cspA* region, lacking the ORF, into an alternative vector. It was possible that *lacZα* region, which is present on pBluescript KS might facilitate unwanted rearrangements through recombination with the *lacZ* reporter gene that was to be inserted within *cspA*. For this purpose, a pBR322 derivative, pBRX, was used as an alternative vector in order to facilitate subcloning, subsequent excision and re-circularisation. In the latter vector, pBR322 was altered, so that the *Hind*III site present at bases 29 - 35 was replaced with *Xba*I. (A *Hind*III site was present in the *cspA-lacZ* fusion (described later) so precluding use of this site in the cloning strategy). Plasmid pBR322 was digested with *Hind*III and the resulting over-hanging termini were end-filled using Klenow polyermase.

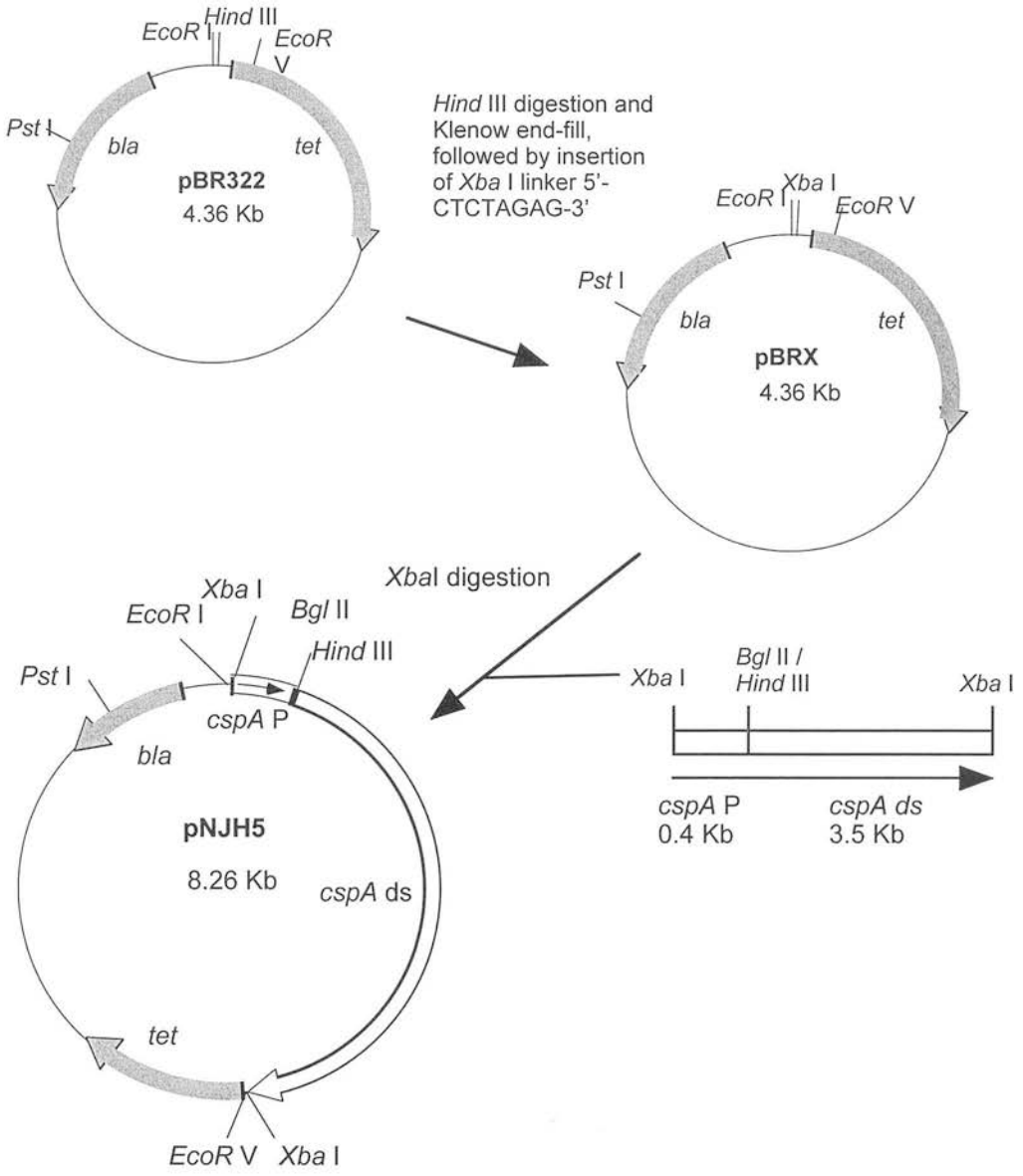


Figure 3.4 A Construction of pBRX and pNJH5.

Plasmid pBR322 was modified so that the *Hind* III site at position 29 - 35 was replaced with *Xba* I. pBR322 was initially digested with *Hind* III and the over-hanging termini were end-filled with Klenow enzyme. A synthetic linker (5'- CTCTAGAG-3'), which contained an *Xba* I site, was then incorporated. Subsequently, a 4.0 Kb fragment containing *cspA* P and *cspA* 3' was subcloned from pNJH1 into pBRX, by *Xbal* i digestion and ligation, forming pNJH5.

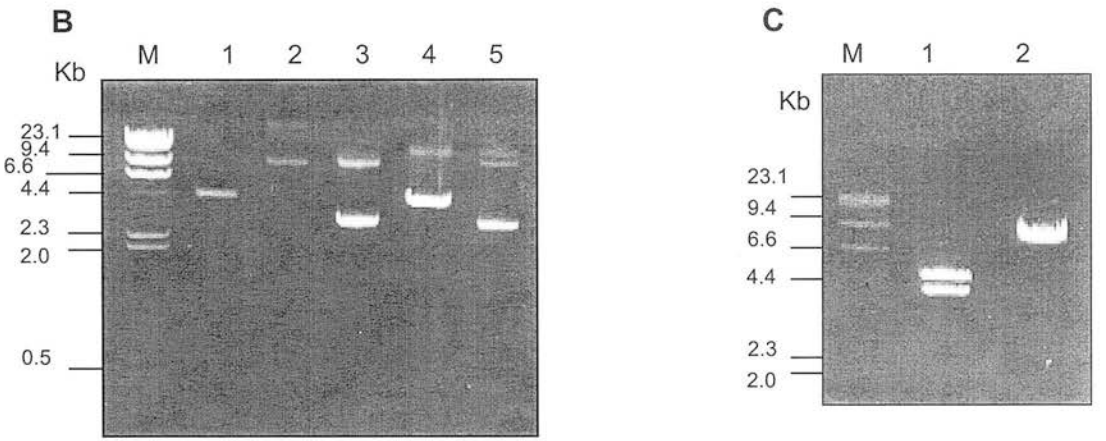


Figure 3.4 B and C: Restriction analysis of pBRX and pNJH5.

3.5 B Lane 1, pBR322 digested with *Hind*III; lane 2, pBR322 digested with *Xba*I; lane 3, pBRX digested with *Hind*III; lane 4, pBRX digested with *Xba*I; lane 5, un-digested pBRX; M, DNA ladder (λ DNA digested with *Hind*III), the sizes and positions have been indicated.
3.5 C Lane 1, pNJH5 digested with *Xba*I; lane 2, pNJH5 digested with *Hind*III; M, DNA ladder (λ DNA digested with *Hind*III), the sizes and positions have been indicated.

A synthetic phosphorylated *XbaI* linker sequence 5'-CTCTAGAG-3', was ligated between the blunt ends to form pBRX, thereby destroying the *Hind*III site (figure 3.4, panel A). The latter plasmid is 2 bp larger than pBR322. Restriction analysis of pBRX confirmed that this procedure was successful (figure 3.4 B). Digestion of the plasmid DNA with *Xba*I produced a 4.4 Kb fragment, whilst incubation of pBRX with *Hind*III did not cut the DNA. Comparative restrictions were made with pBR322.

Once the vector pBRX had been constructed, the truncated *cspA* region was subcloned from pNJH1 by restriction digestion with *Xba*I and inserted into the corresponding site in pBRX, resulting in pNJH5 (figure 3.4, panel A). Restriction analysis confirmed that the truncated *cspA* region was successfully subcloned into pBRX (figure 3.4 C). Digestion with *Xba*I generated a 4.0 Kb fragment containing the *cspA* P region and *cspA* ds region, in addition to a fragment of approximately 4.4 Kb which corresponded to the remainder of the vector.

The next step involved inserting *lacZ* into the artificial *Bgl*II and *Hind*III sites at position + 110 of *cspA* (relative to the transcriptional start site, the translation start site is located at base +156). The *lacZ* gene was PCR amplified from wild type *E. coli* (EMG2) using primers LAC5 and LACZR (table 2.3, chapter 2 of this thesis). The former primer corresponded to bases 1232 – 1262 of the coding strand and incorporated an artificial *Bgl*II restriction site at the 5' end. The latter primer was complementary and anti-parallel to bases 4368 – 4340 of the non-coding strand and incorporated an artificial *Hind*III restriction site at the 5' end (Hediger *et al.*, 1985). The PCR amplified DNA containing the *lacZ* gene was restriction digested with *Bgl*II and *Hind*III, and inserted into the corresponding sites in pNJH5, resulting in pNJH6 (figure 3.5, panel A). The *cspA*P-*lacZ* fusion included a ribosome binding site derived from *lacZ* (EMBL accession number J01636). Restriction analysis confirmed that *lacZ* was successfully cloned into pNJH5 (figure 3.5 B).

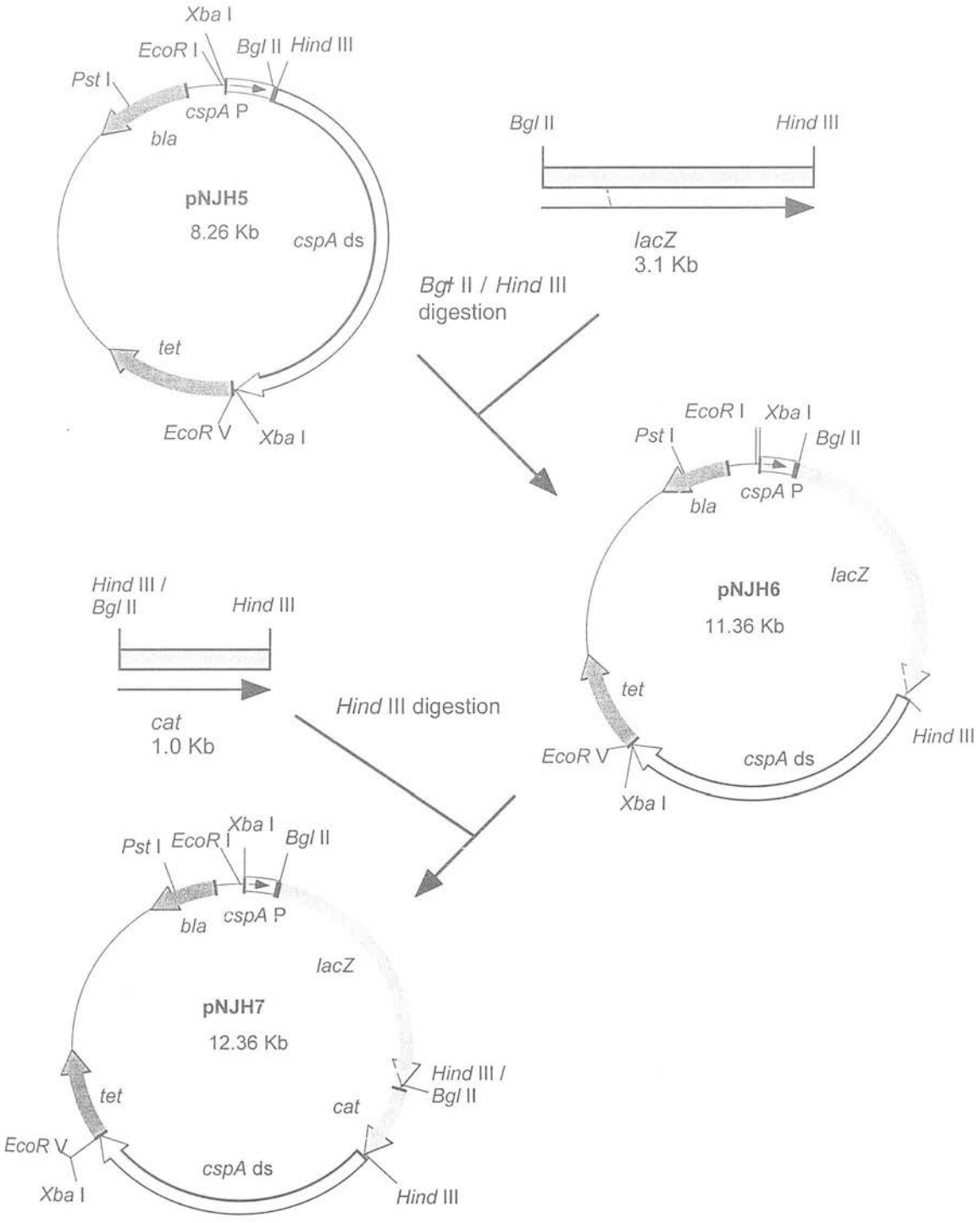


Figure 3.5 A Construction of pNJH6 and pNJH7.
The *lacZ* gene was amplified by PCR from *E. coli* strain EMG2 and cloned into the *Bgl* II and *Hind* III sites of pNJH1, resulting in pNJH6. Subsequently, the *cml* cassette was amplified by PCR from pBR325 and cloned into the *Hind* III site of pNJH6, resulting in pNJH7.

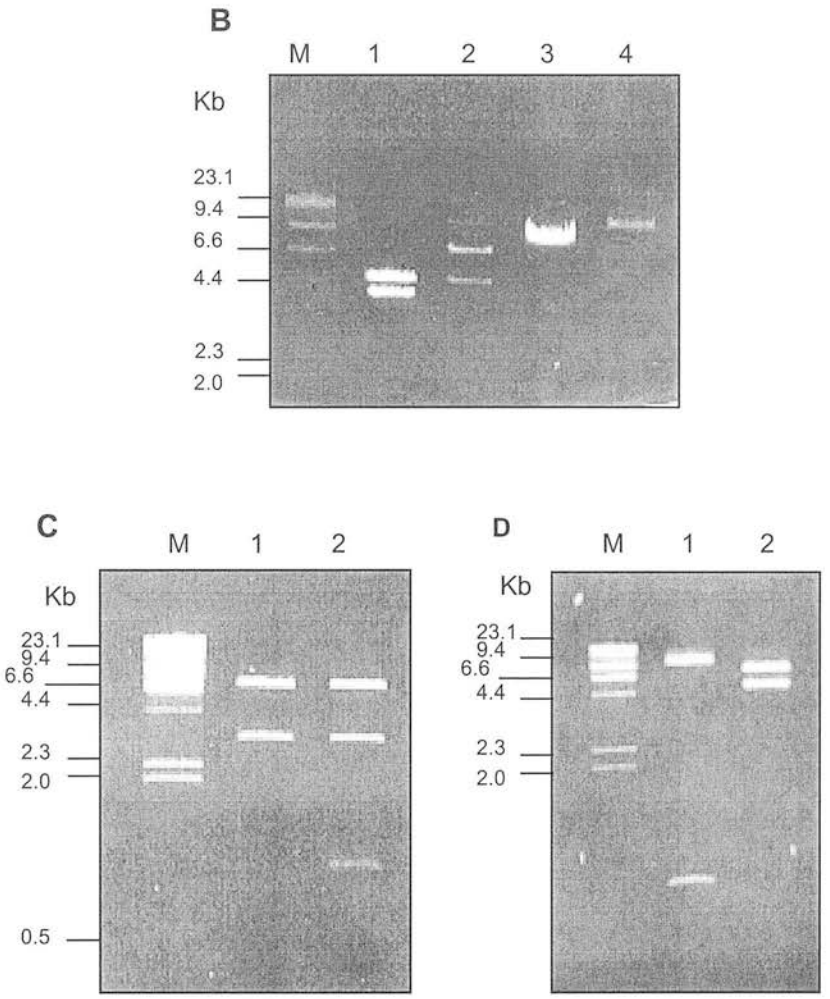


Figure 3.5 B, C + D: Restriction analysis of pNJH6 and pNJH7.

3.5 B. Lane 1, pNJH5 digested with *Xba*I; lane 2, pNJH6 digested with *Xba*I; lane 3, pNJH5 digested with *Hind*III; lane 4, pNJH6 digested with *Hind*III; M, DNA ladder (λ DNA digested with *Hind*III), the sizes and positions have been indicated.

3.5 C. Lane 1, pNJH6 digested with *Hind*III and *Bgl*II; lane 2, pNJH7 digested with *Hind*III and *Bgl*II; M, DNA ladder (λ DNA digested with *Hind*III), the sizes and positions have been indicated.

3.5 D. Lane 1, pNJH7 digested with *Hind*III; lane 2, pNJH7 digested with *Bgl*II; M, DNA ladder (λ DNA digested with *Hind*III), the sizes and positions have been indicated.

Digestion with *Xba*I generated a 4.0 Kb fragment containing the *cspA* P region and *cspA* ds region, in addition to a fragment of approximately 7.5 Kb which corresponded to the *lacZ* DNA and the remainder of the vector. Digestion with *Hind*III generated a single DNA fragment of approximately 11 Kb, which corresponded to the linearised plasmid. Comparative restriction digestions were made with pNJH5.

Subsequently, a *cat* cassette, which included the native promoter and RBS, was inserted downstream of *lacZ*, to confer antibiotic selection on the construct. To achieve this, the *cat* cassette was PCR amplified from pBR325 (Gilbert, unpublished, accession number: L08855) using primers 5'-CML and 3'-CML (table 2.3, chapter 2 of this thesis). The former primer corresponded to bases 4288 – 4312 of the coding strand of *cat* on pBR325 and incorporated an artificial *Hind*III restriction site at the 5' end, while the latter primer was complementary and anti-parallel to bases 5262 – 5234 of the non-coding strand of *cat* on pBR325 and also incorporated an artificial *Hind*III site at the 5' end, in addition to a *Bgl*II site. The PCR amplified *cat* cassette was inserted into the *HindIII* site of pNJH6, resulting in pNJH7 (figure 3.5, panel A). The plasmid pNJH7 was found to be resistant to chloramphenicol. Restriction analysis confirmed that the *cat* cassette was successfully cloned into pNJH6 (figure 3.5 C). Digestion with *Hind*III and *Bgl*II generated fragments of 1.0, 3.1 and 8.15 Kb, which corresponded to the *cat* cassette, *lacZ* DNA and the remainder of the plasmid, respectively. The orientation of the *cat* cassette was determined by restriction analysis with *Bgl*II and found to be in the same orientation as *cspA* and *lacZ* (figure 3.5 D).

3.2.3 Allelic recombination of *cspA-lacZ-cat* with the *S. typhimurium cspA* locus.

Attempts were made to recombine the modified *cspA* construct of pNJH7 into the chromosome of *S. typhimurium* (SL1344) by allelic exchange, as shown diagrammatically in figure 3.6.

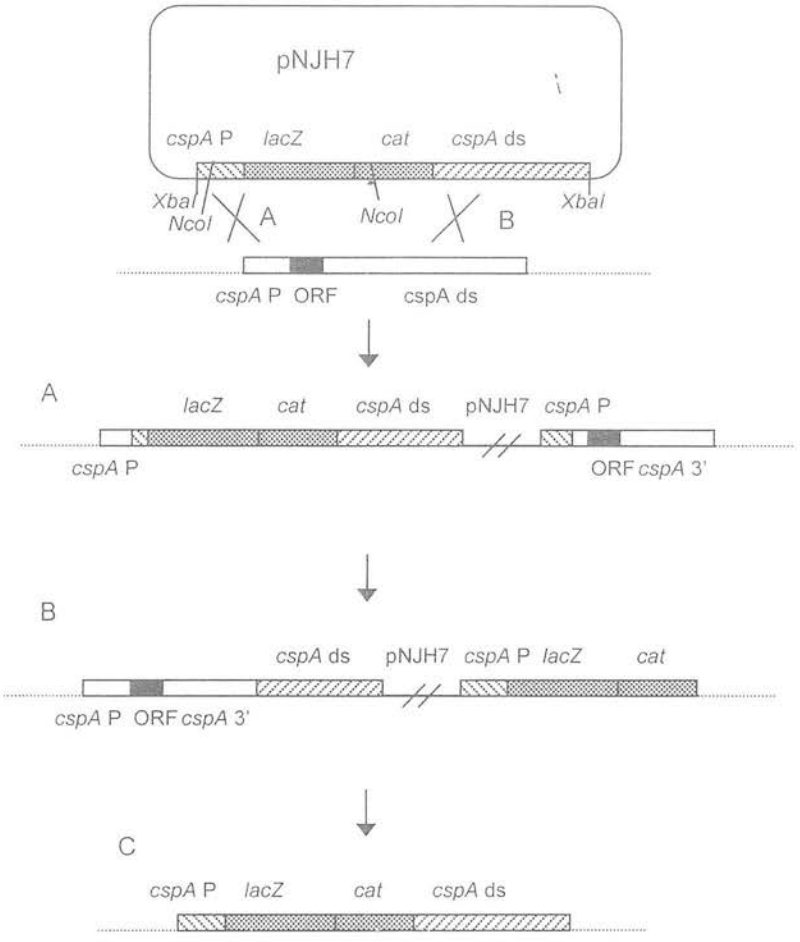


Figure 3.6 Construction of a *S. typhimurium cspA-lacZ* strain.

Integration of the *cspA-lacZ* *Xba*I fragment by homologous, single cross-over recombination events. Both possible single recombination outcomes are indicated here; recombination upstream of the *cspA* ORF (A), and recombination downstream of the *cspA* gene (B). In addition, the result of a double cross-over event has been shown (C). The plasmid is indicated by solid lines and the chromosome is indicated by dotted lines. The open boxes indicate the chromosomal *cspA* gene with the promoter, P, and downstream region, ds, respectively. The solid box indicates the *cspA* ORF. The hatched boxes indicate the disrupted *cspA* gene on the plasmid, and the shaded box indicates the *lacZ* gene. (Not drawn to scale).

For this purpose, initially, the *cspA-lacZ-cat* sequence was excised from pNJH7 by restriction digest with *Xba*I and re-circularised by ligation. The circularised construct was then introduced into competent SL1344 cells by electroporation. The cells were allowed to recover at 37°C for 1 hour in LB medium, with shaking, and recombinants were selected for on agar supplemented with chloramphenicol. Unfortunately, these attempts proved unsuccessful and no transformants were isolated.

As an alternative approach to facilitate recombination, a *polA* strain (CH607) was used for transformation. The *polA* gene encodes DNA polymerase I and mutation of this gene leads to loss of replication ability of ColE1 based plasmids. The *cspA-lacZ-cat* construct was prepared in the same manner as before and introduced into competent cells of the LT2-derived *polA* mutant, CH607, by electroporation. Approximately 50 ng of circular DNA was used to transform 1 x 10^9 competent cells. The cells were allowed to recover for 1 hour in LB at 37°C, with shaking, before plating out 0.2 ml of culture. Approximately 200 colonies were subsequently recovered on agar containing chloramphenicol. The Cml^R colonies were then tested for ampicillin resistance by streaking on parallel agar plates, containing either ampicillin or no antibiotic supplement. 25 colonies were found to be sensitive to ampicillin while the remaining 175 colonies were resistant. This suggested that the Amp^R colonies harboured the plasmid pNJH7, which may have been inadvertently integrated onto the chromosome via a single cross-over event, during the attempts to introduce the *cspA-lacZ-cat* into CH607. However, it was also possible that the host strain may have reverted to $polA^+$ which would lead to replication and maintenance of ColE1 based plasmids.

The chromosomes of the Cml^R Amp^S colonies were analysed by Southern blotting to distinguish between the different types of recombination events (figure 3.7). The four possible outcomes of recombination are as follows: no recombination; a double cross-over event where complete allelic recombination is successful; or either of 2 single cross-over events. In the latter cases, integration of the disrupted copy of the gene occurs either upstream or downstream of the native gene (figure 3.6).

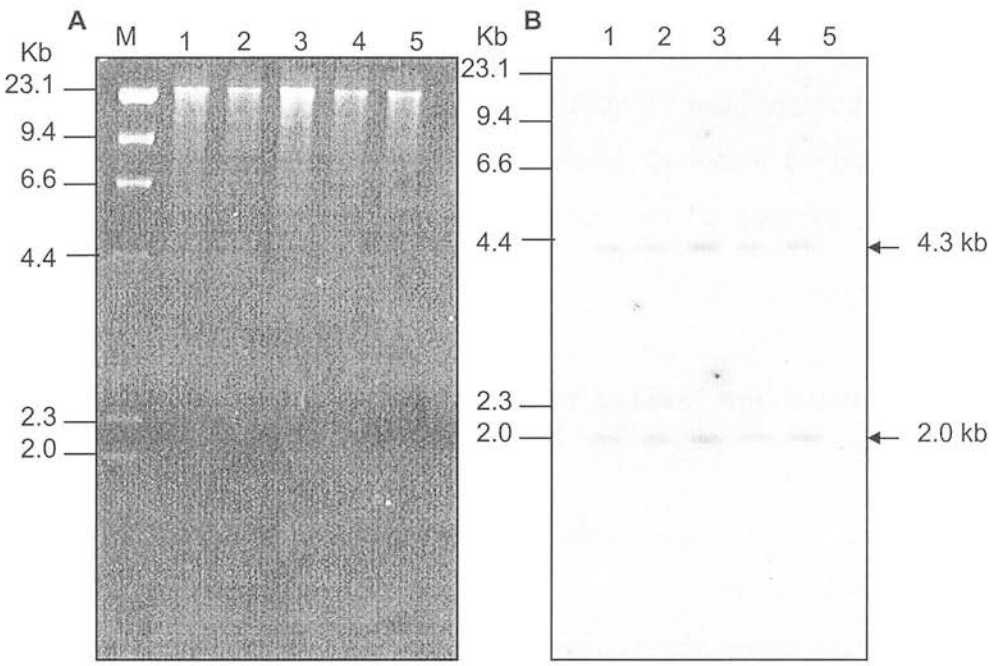


Figure 3.7 Southern blot of DNA from isolates of CH607.

Analysis of genomic DNA from isolates of CH607 with possible *cspA* disruptions. Southern blot showing genomic *Nco*I digests, probed with *cspA* DNA from base 1 - 370.
Panel A; agarose gel. Panel B; corresponding autoradiograph. Lanes 1 to 5 correspond to samples 1 to 5. The sizes (Kb) and positions of the DNA markers are shown (λ digested with *Hind*III).

The DNA was isolated from the colonies, digested with *Nco*I, Southern blotted and probed with ^{32}P-labelled DNA which corresponded to bases –264 to + 125 of *cspA*. In plasmid pNJH7, the *Nco*I sites are positioned at base 27 of *cspA* and at base 5080 of *cat*, which yields a fragment of 4.3 Kb from the *cspA-lacZ-cat* construct. Southern blots showed that in addition to the 4.3 Kb band of the disrupted *cspA*, a 2.0 Kb band was also evident. Figure 3.7 shows the results for 5 of the recombinants. These results suggested that only a single recombination event had occurred, as depicted in figure 3.6, and the size of the fragments suggested that recombination had occurred downstream of the native *cspA* gene. The presence of the 2nd band indicated that in addition to the disrupted copy of *cspA* on the chromosome, the native, complete copy of *cspA* was also present, therefore these strains could not be used for mutation studies.

3.2.4 Construction of a *cspA-cat* reporter system and subsequent attempts at allelic recombination.

One explanation for the failure to isolate a mutant carrying the disrupted *cspA* gene may have been that the large size of the *lacZ-cat* insert reduced the probability of a double cross-over event occurring between the flanking *cspA* sequences and the chromosome. As an alternative approach, the promoter-less *cat* gene from pCM4 was inserted at position + 135 of *cspA* to provide a means of positive selection, following recombination, and also to serve as a reporter. The *cspA-cat Xba*I fragment was only 4.7 Kb compared to the 8.0 Kb *XbaI* fragment of *cspA-lacZ-cat*. It was thought that this would improve electroporation efficiency and also the probability of recombination. The *cat* gene was sub-cloned using the native *Bam*HI sites at the 5' and 3' ends of the gene, from pCM4 into the *Bgl*II site of pNJH1. The resulting plasmid was termed pNJH8 (figure 3.8, panel A). Restriction digestion analysis was carried out to confirm that the *cat* gene had been successfully cloned into pNJH1 (figure 3.8 B). Digestion with *Nco*I generated fragments of 0.9, 1.4 and 5.3 Kb, due to 3 *Nco*I sites present in *cspA* P (located 25 bp from the 5' end), *cat* (located 500 bp from the 5' end) and in the *cspA* ds region (approximately 1.2 Kb from the 5' end).

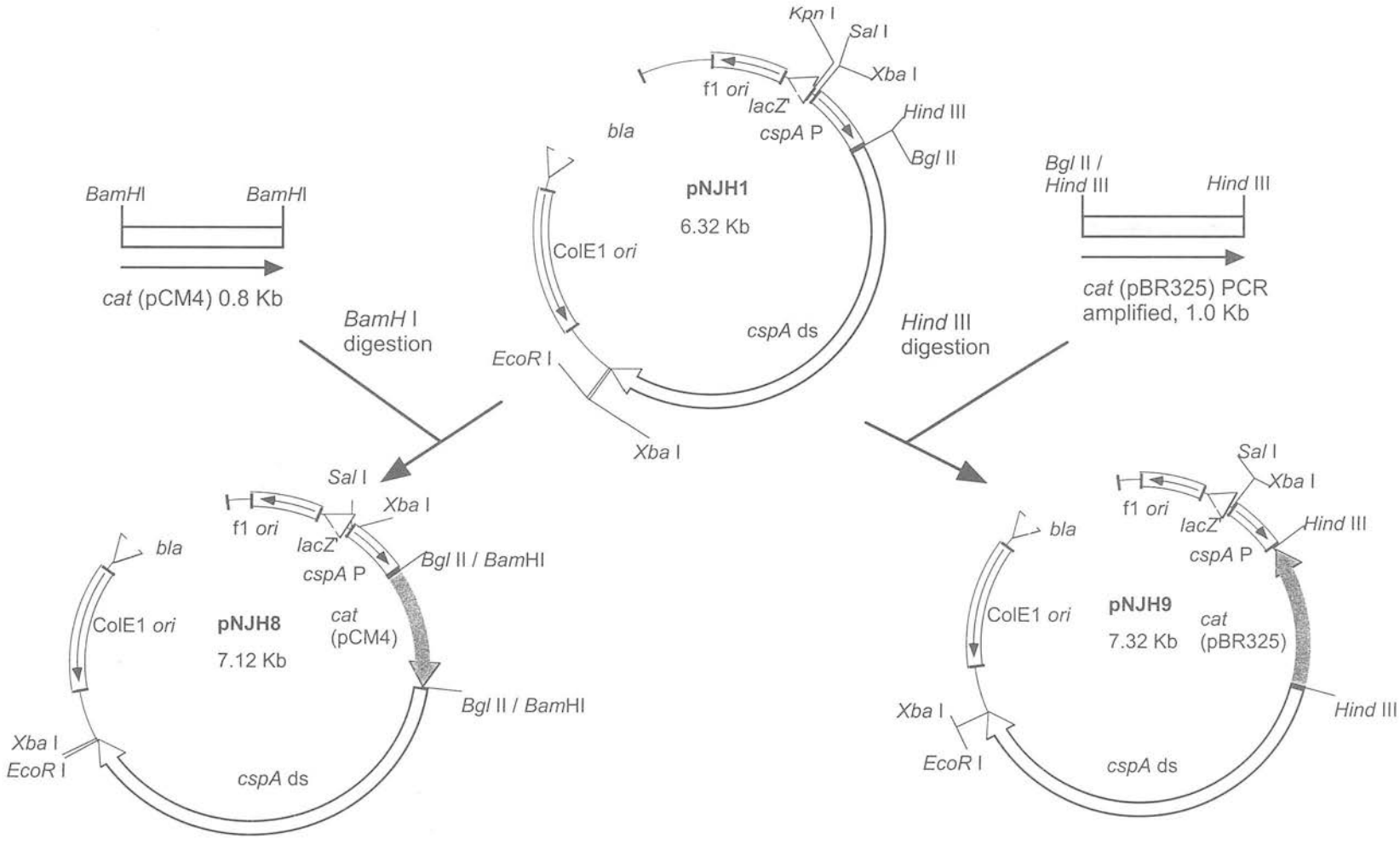


Figure 3.8 A Construction of pNJH8 and pNJH9.

The promoterless *cat* gene was subcloned from pCM4 into the *Bgl* II site of pNJH1, resulting in pNJH8. As an alternative, the *cml* cassette complete with promoter region, was amplified by PCR from pBR325 and cloned into the *Hind* III site of pNJH1, resulting in pNJH9.

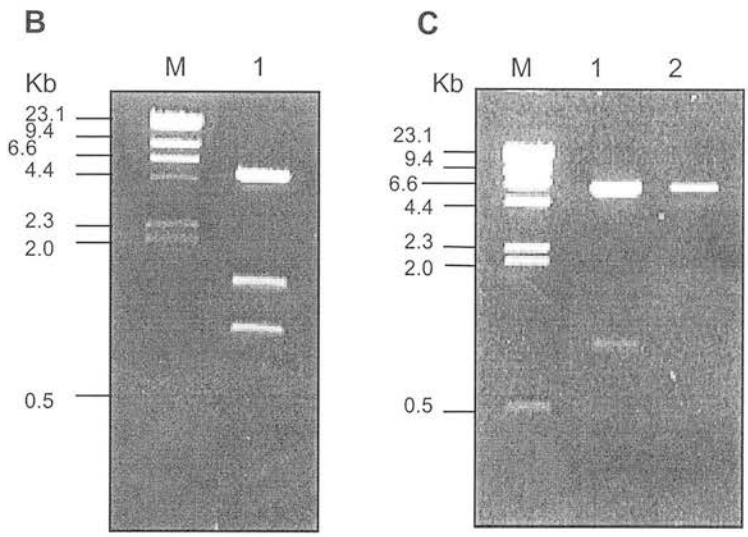


Figure 3.8 B + C: Restriction analysis of pNJH8 and pNJH9.

3.8 B. Lane 1, pNJH8 digested with *Nco*I; M, DNA ladder (λ DNA digested with *Hind*III), the sizes (in Kb) and positions have been indicated.

3.8 C. Lane 1, pNJH9 digested with *Bgl*II; lane 3, pNJH1 digested with *Bgl*II; M, DNA ladder (λ DNA digested with *Hind*III), the sizes and positions have been indicated.

Subsequently, the *cspA-cat* fragment was removed from pNJH8 by restriction digesting with *Xba*I, and the fragment was circularised by ligation and introduced into competent cells of SL1344 or CH607 by electroporation. Approximately 50 ng of circular DNA was transformed into 1 x 10^9 competent cells. The cells were allowed to recover at 37°C in 1 ml of LB for 1 hour with shaking, and 0.2 ml of cells were plated on LB agar containing chloramphenicol. However, no colonies were recovered. It has been reported by Fang and colleagues (1997) that *E. coli cspA* mRNA is extremely unstable at 37°C. This fact together with the absence of any chloramphenicol resistant recombinants suggested the possibility that the *cspA-cat* mRNA may not be stable at 37°C. Thus, such cells would be sensitive to chloramphenicol at this temperature. To circumvent this possibility, the cells were allowed to recover for 2 hours in LB media at 23°C, a temperature which *cspA* is more likely to be expressed (Etchegaray *et al.*, 1996). In addition, colonies were grown on agar containing chloramphenicol at 23°C. Unfortunately, no recombinants were recovered following selection in this manner either.

3.2.5 Disruption of *cspA* by inserting a constitutively expressed *cat* cartridge.

3.2.5.i Strategy 1 – *cspA-cat* from pNJH10

As an alternative to using the promoter-less *cat* gene under the control of *cspA*, *cspA* was disrupted with a complete *cat* cassette from pBR325, which contained the *cat* promoter (figure 3.8 A)

Although *cspA* gene expression could not be monitored, this strategy would still allow insertional inactivation of chromosomal *cspA*. The *cat* cassette was PCR amplified, as described above for pNJH7, using primers CML-5 and CML-3 (table 2.3, chapter 2 of this thesis). The *cat* cassette was inserted into the *Hind*III site of pNJH1, resulting in pNJH9 (figure 3.8 A). Restriction digestion analysis confirmed that the *cat* cassette had been successfully cloned into pNJH1 (figure 3.8 C). Digestion with *Bgl*II produced a 1.0 Kb fragment which corresponded to the *cat*

cassette, and a 6.1 Kb fragment which corresponded to the remainder of the plasmid, confirmed by comparative digestion of pNJH1.

In addition to insertion of the *cat* cassette, the 3' region downstream of *cspA* was truncated from 3.5 kb to a length of approximately 1.5 kb long. This strategy was used to encourage double recombination events by reducing the size of the homologous DNA which was available for recombination at the 3 end. This was carried out as follows: restriction analysis revealed that plasmid pNJH9 contained 1 *Pst*I site within the *cspA* ds region, approximately 1.5 kb downstream from the end of the *cat* cassette. The region containing *cspA* P, the *cat* cassette and *cspA* ds was removed from pNJH9 by restriction digestion with *Xba*I and *Pst*I, and was sub-cloned into pBRX using the newly engineered *Xba*I site (base 29) and the *PstI* site within the *bla* gene (base 3607). The new plasmid, termed pNJH10, was Cml^R and Amp^S, since part of the *bla* gene had been deleted during the sub-cloning procedure (figure 3.9 A). Restriction digestion confirmed that the subcloning step was successful (figure 3.9 B). pNJH10 was digested with enzymes, *Pst*I, *Xba*I and *BamH*I, to generate a single fragment which corresponded to the whole plasmid, and *Hind*III which produced 1.0 Kb and 5.4 Kb fragments, corresponding to the *cat* cassette and the remainder of the plasmid, respectively.

Insertion of the *cspA-cat* fragment onto the chromosome by allelic recombination was attempted at 37°C, as described previously for the *cspA-lacZ-cat* sequence in cells of CH607. It was thought that a double recombination event would occur, as shown diagrammatically in figure 3.10. Following electroporation of the re-circularised DNA fragments, the cells were allowed to recover in LB for 1 hour at 37°C, before 0.2 ml of the cells were plated onto LB agar containing chloramphenicol. However, no recombinants were recovered. The efficiency of competence of the CH607 cells was tested by also transforming 300 ng of pNJH10 plasmid, which resulted in recovery of more than 160 chloramphenicol resistant isolates per 0.2 ml of culture.

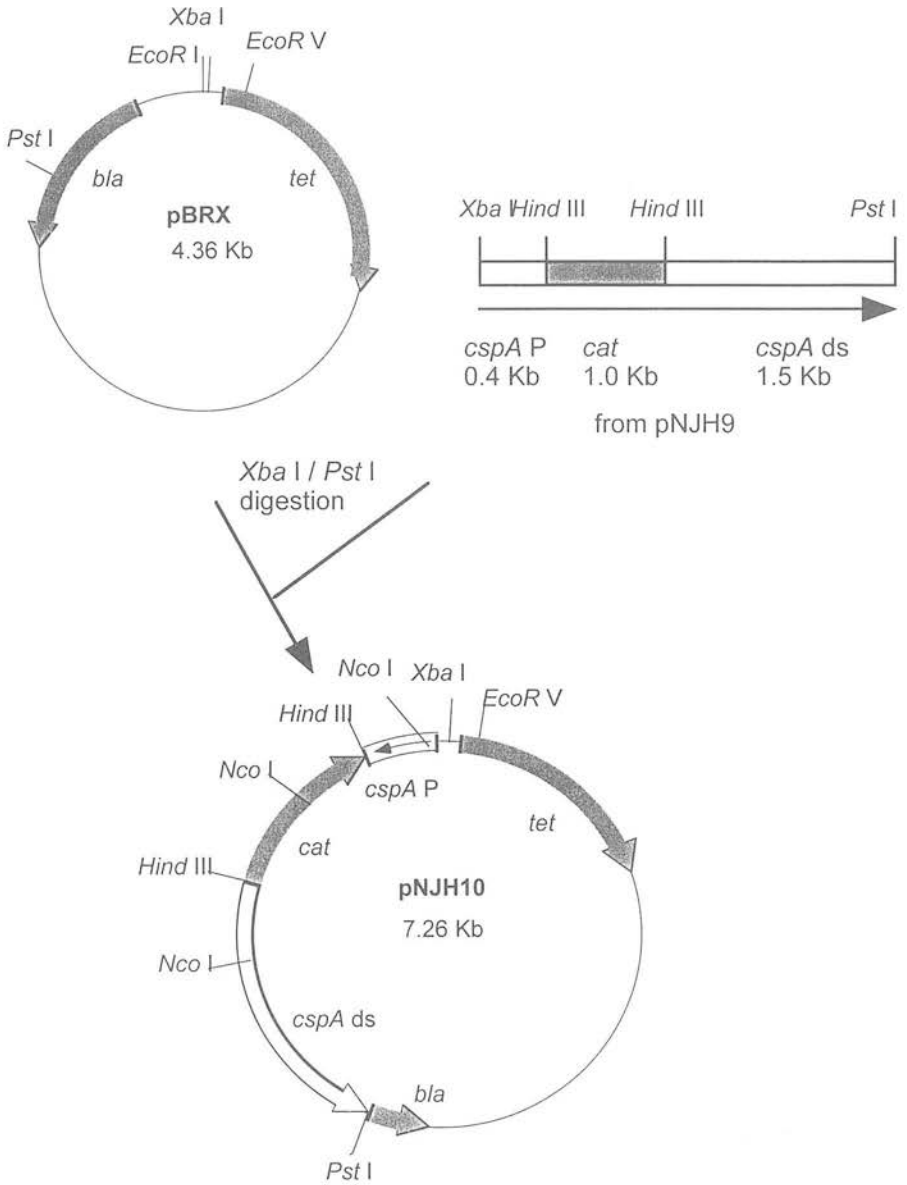


Figure 3.9 A Construction of pNJH10.

A fragment containing the *cspA* P region, the *cml* cassette and the *cspA*'ds region was subcloned into pBRX, by digestion of pNJH9 with *Xba* I and *Pst* I and subsequent cloning into the compatible sites in pBRX, forming pNJH10.

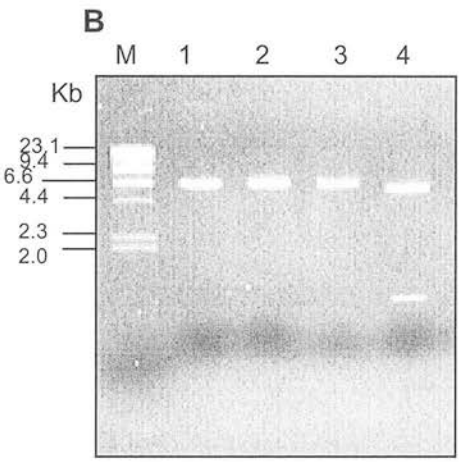


Figure 3.9 B Restriction analysis of pNJH10.

Lane 1, pNJH10 digested with *Xba*I; lane 2, pNJH10 digested with *Pst*I; lane 3, pNJH10 digested with *BamH*I; lane 4, pNJH10 digested with *Hind*III; M, DNA ladder (λ DNA digested with *Hind*III), the sizes and positions have been indicated.

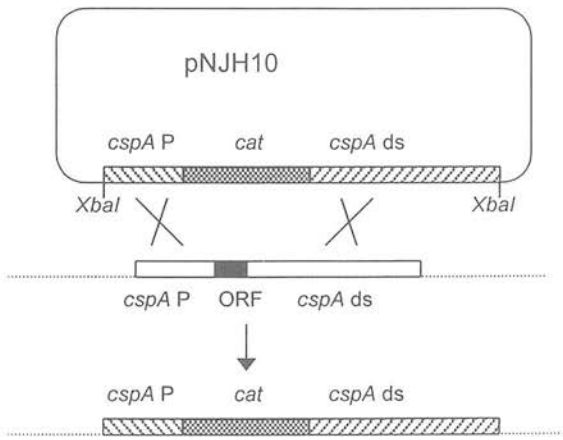


Figure 3.10 Allelic exchange of *S. typhimurium cspA* with *cspA-cat* DNA.

Recombination strategy for integration of the *cspA-cat Xba*I fragment into the chromosomal *cspA* locus by homologous recombination. The plasmid is indicated by solid lines and the chromosome is indicated by dotted lines. The open boxes indicate the chromosomal *cspA* gene, the promoter, P, and downstream region, ds, respectively. The solid box indicates the *cspA* ORF. The hatched boxes indicate the disrupted *cspA* gene on the plasmid, and the shaded box indicates *cat*. (Not drawn to scale).

Whether this level of competence was insufficient to allow recombination of the *cspA-cat* fragment remains unclear. An alternative strategy was utilised to increase the likelihood of successful recombination.

3.2.5.ii Strategy 2 – PCR amplification of *cspA-cat* from pNJH10 and sequence of the region downstream of the *cspA* ORF.

A second plasmid was constructed to attempt allelic recombination containing the *cspA-cat* sequence. For this strategy, the region downstream of the *cspA* ORF was reduced so that it was a similar length to the region upstream of the *cspA* ORF. It was thought that by having similar lengths of flanking *cspA* DNA, the chances of a double recombination event would increase. The shorter version of the *cspA-cat* DNA was amplified by PCR from pNJH10 and introduced in SL1344 by electroporation, as described below.

3.2.5.iii DNA sequence downstream of the *cspA* ORF.

The region downstream of the *cspA* ORF was unknown, so it was sequenced from the end of the ORF, using an automatic ABI Prism™ DNA sequencer (Applied Biosystems). A primer, CSPA1 (table 2.3, chapter 2 of this thesis) was designed that corresponded to bases 576 – 594 of *cspA* (figure 3.1). The plasmid, pJEC17 that contains the entire *cspA* sequence, was prepared by a modified alkali lysis and PEG precipitation procedure (described in the Methods chapter of this thesis) so that it was free of associated proteins and salts. The CSPA1 primer and the BigDye™-terminator reaction mix were added to the plasmid and the sequencing reaction was carried out over 25 cycles as follows: 96°C for 30 seconds, 50°C for 20 seconds and 60°C for 4 minutes. The extension product was ethanol precipitated and reconstituted in a loading buffer. The sample was then heat denatured and electrophoresed. Over 600 bp were successfully sequenced (figure 3.11). This sequencing reaction was only carried out once, on the coding strand, therefore it is possible that errors in the sequence may be present. However, the main objective of obtaining the sequence was to design a primer (CSP6) approximately 500 bp downstream of the stop codon of *cspA* ORF (figure 3.11, underlined).

to design a primer (CSP6) approximately 500 bp downstream of the stop codon of *cspA* ORF (figure 3.11, underlined).

The DNA sequence was analysed using a FastA Align programme (Pearson, 1996), to determine whether it contained any known open reading frames. This analysis revealed a putative ORF, which started 180 bp after the *cspA* stop codon. The putative ORF was in the same orientation as *cspA* and started with an ATG codon. The derived amino acid sequence was subsequently found to share 24.5 % sequence identity to a 139 amino acid overlap of an alkylmercury lyase from *Staphylococcus aureus* encoded by *merB* (Laddaga *et al.*, 1987). The amino acid sequence of the putative ORF has been aligned to the *S. aureus* MerB protein (figure 3.12).

Bacterial resistance to mercurials has been well studied at both the genetic and biochemical level. Resistance to inorganic mercury compounds is conferred by the narrow spectrum mercury resistance (*mer*) operons, which consist of a regulatory gene (*merR*), an operator/promoter region and a polycistronic operon of structural genes (frequently *merT*, *merA* and *merP*). The broad spectrum mercury resistance operons are generally the same as the narrow range operons, with the addition of *merB*, which specifies the organomercurial lyase enzyme (Kiyono *et al.*, 1997). The operons that encode mercury resistance are plasmid based and are most commonly reported from studies of *Pseudomonas* strains and *Staphylococcus aureus* (Silver & Walderhaug, 1992).

Comparison of the amino acid sequence of MerB from several *Pseudomonas* plasmids and *Bacillus* species has shown that 3 cysteine residues are conserved in this group (MerB homologues from pMRA17, pDU1358, pPB117, pI258 and *Bacillus* species). The position of these residues has been indicated in figure 3.12, at positions 100, 121, 162 of *S. aureus* MerB. Only the cysteine residue at position 121 of MerB is conserved in the putative ORF from *S. typhimurium* (figure 3.12). This suggests that the level of homology is not high and consequently, there is a possibility that the putative ORF is not a *merB* homologue.

```
621   TAACCAGCCT GTaagcttAA AAGCTCAGCA TTTTGATCCT GCTGATGGCG
          T  S  L   *

671   GGATTTTTTT TATTCCAATT CCCCCCTCTC CCCCAGCATA ACTTTGCATT

721   ACTTTACCCT GCGTCCCTTT GACCTTTCCC TTAGGGGAAC CCCTATAGTA

771   GGCAGGGAGA TTGTTCACAA GGAATTGAAG TTatgTCGAA ATCATCATGG
                                          M  S  K  S  S  W

821   TTATTACTTT TGGGTTTATG CGCCAGCGGC TCCGCGCTTG CGGCATCCTC
        L  L  L  L  G  L  C  A  S  G  S  A  L  A  A  S

871   AGAATCCGCT TTTCTGGCGC AACATGGGCT GGCGGGAAAA ACGGTTGAGC
      S  E  S  A  F  L  A  Q  H  G  L  A  G  K  T  V  E

921   AGATCGTGGA TACGATTGAT CAGACGCCGC AGAGCCGTCC TTTGCCTTAC
      Q  I  V  D  T  I  D  Q  T  P  Q  S  R  P  L  P  Y

971   TCCGCGTCGA TTACCAGTAC CGAACTCAAA TTATCTGACG GAGAGCAGAT
        S  A  S  I  T  S  T  E  L  K  L  S  D  G  E  Q

1021  TTATACGCTG CCGTTAGGCG ACAAATTCTA TCTCTCTTTT GCGCCCTATG
      I  Y  T  L  P  L  G  D  K  F  Y  L  S  F  A  P  Y

1071  AATGGCGGAC ACACCCCTGT TTTAACCACA GCCTTTCCGG CTGTCAGGGA
      E  W  R  T  H  P  C  F  N  H  S  L  S  G  C  Q  G

1121  GAAATGCCGA ATAAACCGTT CACTGTAAAA GTGACAGACA GTAAAGGCGC
      E  M  P  N  K  P  F  T  V  K  V  T  D  S  K  G  A

1171  TGTCATCGTA CAAAAAGAGA TGCAAAGCTA TCGAAACGGA TTTATCGGTG
        V  I  V  Q  K  E  M  Q  S  Y  R  N  G  F  I  G

1221  TTTGGCTACC GCGCAATATG GAAGGGACGC TGGAAGTAAG CTACAACGGT
      V  W  L  P  R  N  M  E  G  T  L  E  V  S  Y  N  G

1271  AAAACGGCGT CACATGCNAT TGCCACCAGT
      K  T  A  S  H  A  I  A  T  S
```

Figure 3.11 DNA downstream of the *S. typhimurium cspA* gene.

DNA sequence downstream of *S. typhimurium cspA*. The last 4 codons of *cspA* are shown, together with the *Hind*III site located at the stop codon. The base numbers correspond to the sequence in figure 3.3. The underlined sequence indicates the position of the primer CSPA6. The location of the start codon of the putative ORF is shown in lower case and the derived amino acid sequence of the ORF has also been shown.

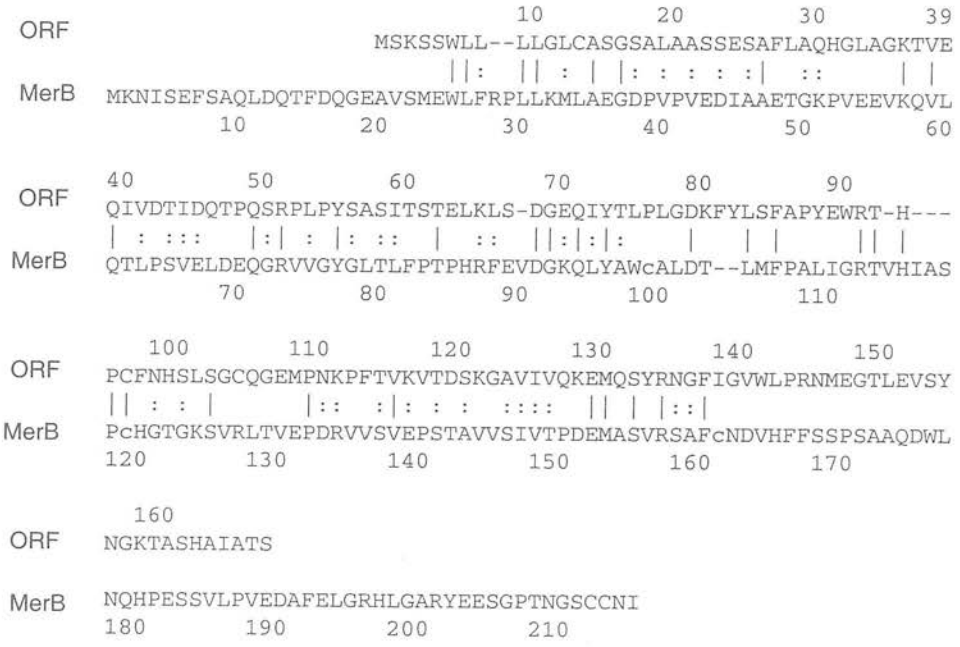


Figure 3.12 Alignment of putative ORF downstream of *S. typhimurium cspA* with *S. aureus* MerB.

The sequences were aligned using the FastA Align programme (Pearson, 1996), The best alignment of the putative ORF was with a region of 139 amino acid residues of MerB. Cysteine residues that are conserved in the MerB proteins of other bacteria have been indicated in lower case. The vertical bars (|) indicate identical amino acids, the colons (:) indicate conserved substitutions. The horizontal bars (-) between the amino acid residues indicate gaps in sequence alignment.

The new sequence allowed construction of the primer, CSPA6 (table 2.3 chapter 2 of this thesis), which was complementary to bases 1142 – 1119 of the non-coding strand of the region downstream of *cspA* and incorporated an artificial *Sal*I restriction site at the 5' end. Primer CSPA6 was used in conjunction with MICG1 (described above) to amplify by PCR, a fragment of 1.92 Kb in length, containing *cspA* P (420 bp long), the *cat* cassette (1 Kb long) and *cspA* ds (500 bp long) from pNJH10. The fragment was digested with *Sal*I and circularised by ligation. The circularised *cspA-cat* fragment was introduced into competent SL1344 by electroporation, as described above. The cells were plated on agar containing chloramphenicol at 37°C. Unfortunately no recombinants were recovered in this manner. As before, the efficiency of electroporation of SL1344 was tested by introducing plasmid pNJH10. This resulted in the recovery of approximately 400 chloramphenicol resistant transformants per 0.2 ml of culture. Since this level of transformation efficiency is relatively low, it is possible that transformation into *S. typhimurium* SL1344 derivatives is a limiting factor in recombination attempts.

As an alternative host for recombination, a strain containing a *recD* mutation was employed. Previous reports have demonstrated that the *recD* gene product is mainly responsible for degradation of linear DNA (Russel *et al.*, 1989). Using a strain that tolerates linear DNA greatly increases the survival of the DNA following introduction into the cell and also increases the likelihood of recombination.

A strain MPG490 (*recD::*Tn10(Tc^R) which is restriction negative and modification positive) was used for recombination (table 2.1, chapter 2 of this thesis). The *cspA-cat* fragment was amplified by PCR from pNJH10, using primers MICG1 and CSPA6 (table 2.3, chapter 2 of this thesis) and the linear DNA was introduced into competent MPG490 by electroporation. Approximately 500 ng of linear DNA was electroporated into 1 x 10^9 competent cells. The cells were allowed to recover as described earlier. In this case, 18 colonies were recovered on agar containing chloramphenicol. The DNA was isolated from these colonies and digested with *Eco*RI, since there are known *Eco*RI sites approximately 400 bp upstream and 3.5 kb

downstream of the *cspA* ORF on the *S. typhimurium* chromosome, respectively. In addition, there is an *EcoR*I site within the *cat* cassette (base 4779 of pBR325). Assuming a double cross-over event had occurred on the chromosome, digestion with *EcoR*I should release a 900 bp fragment (containing the *cspA* P region and part of the *cat* cassette) and a 4.0 Kb fragment (containing the remainder of the *cat* cassette and part of the *cspA* ds region). The *EcoR*I digested chromosomal DNA was Southern blotted and probed with ^{32}P-labelled DNA corresponding to the *cspA-cat* DNA from base 1 of *cspA* to base 1142 of *cat* (figure 3.13). The DNA probe was amplified by PCR from pNJH10 using primers MIGC1 and CSPA6.

Chromosomal DNA from MPG490 and pNJH10 were also digested with *EcoR*I and Southern blotted, as positive controls. For 6 of the samples, the probe bound to 2 fragments, of approximately 900 bp and 6 Kb in length, (samples 1 – 6 in figure 3.13 A) In addition, the ^{32}P-labelled DNA probe bound to a fragment of approximately 6 Kb in sample 16 and to DNA that was presumably undigested in samples 13 and 14 (figure 3.13 B). The level of radioactivity from these samples was as intense as the plasmid control (lane 11 figure 3.13 B1, and lane 21 figure 3.13 B2), which suggested that the probe had bound to a multicopy plasmid fragment rather than a chromosomal fragment.

The chromosomal DNA from samples 1 – 6 was also digested with *Nco*I. There are 2 *Nco*I sites in *cspA*, at bases 35 and 1050, and a site within the *cat* cassette, at base 5080 of pBR325. Again, if a double cross-over event had occurred, digestion with *Nco*I should release fragments of 600 bp and 1.8 Kb. The *Nco*I digested DNA was Southern blotted and probed with ^{32}P-labelled DNA derived from the *cat* cassette or derived from the *cspA* P region, (figure 3.14, C and D). The *cat* probe was amplified by PCR from pNJH10 using primers CML-5 and CML-3 (from bases 4288 to 5234 of pBR325), and the *cspA* P probe was PCR amplified using primers MICG1 and MICG2 (from bases 1 to 380). Chromosomal DNA from MPG490 and pNJH1 were also digested with *Nco*I and Southern blotted, as positive controls.

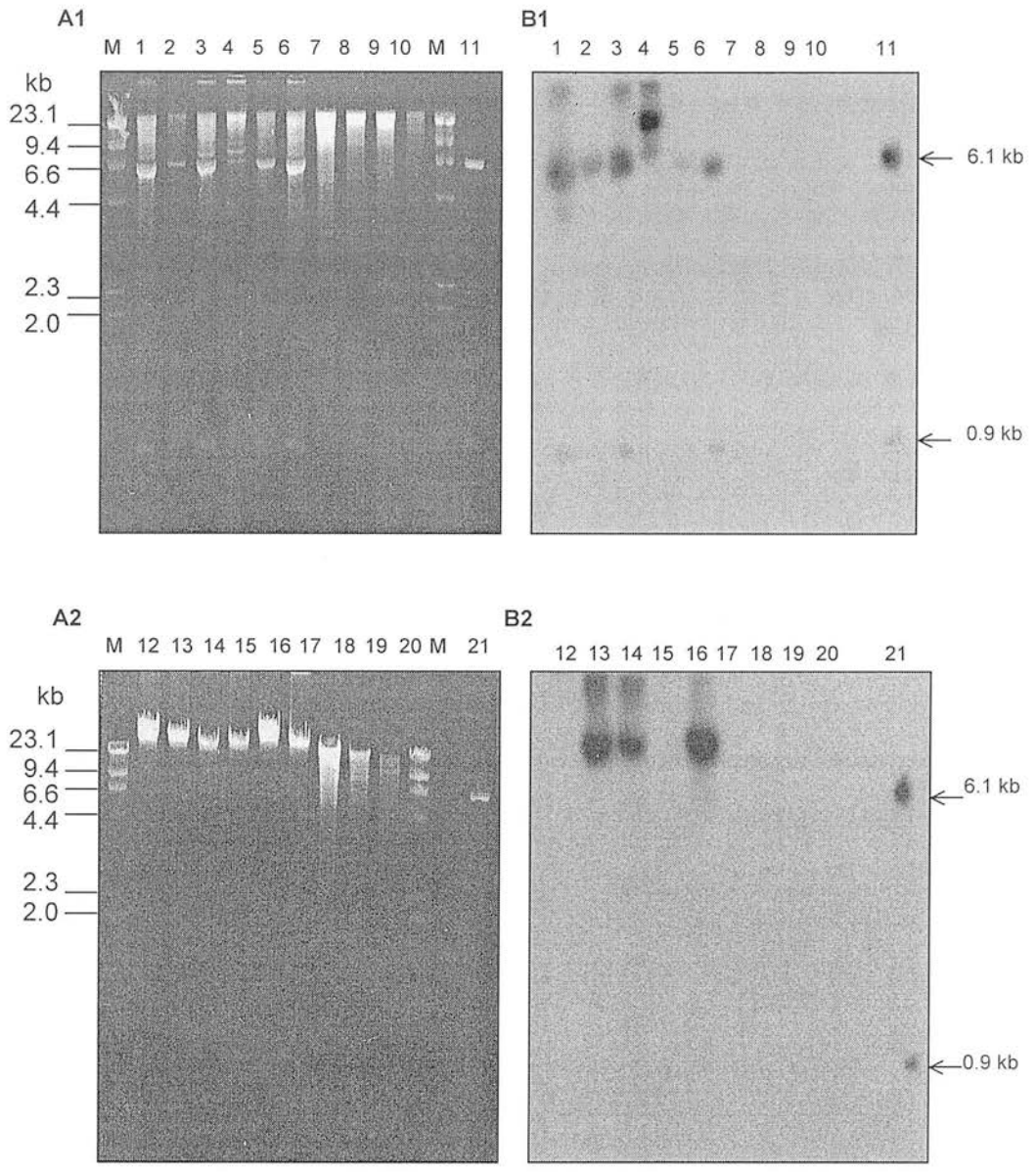


Figure 3.13 Southern blot analysis of genomic DNA from isolates of MPG490.

Analysis of chromosomal DNA from electroporated isolates of MPG490 with possible *cspA* disruptions. Southern blot showing genomic *EcoR*I digests, probed with *cspA-cat* DNA PCR amplified from pNJH10 using primers MICG1 and CSPA6.
3.14 A.1 and A2 - DNA agarose gels showing levels of DNA loaded.
3.14 B.1 and B2 - Corresponding autoradiographs. The positions of the DNA bands of interest have been indicated with arrows.
Lanes 1 to 9 and 12 to 19 correspond to samples 1 to 9 and samples 10 to 17, respectively. Lanes 10 and 20 contain MPG490 DNA. lanes 11 and 21 contain pNJH10 DNA. M is the λ DNA *Hind*III ladder, with positions and sized (in kb) shown.

Following hybridisation with the *cat* probe, the level of radioactivity from DNA fragments of approximately 600 bp and 1.9 Kb was particularly high for sample 1 to 6 (figure 3.14 B). This was similar to the situation found with the *EcoR*I digested DNA. In addition, following hybridisation with the *cspA* probe, the level of radioactivity from DNA fragments of approximately 600 bp and 4.5 kb was almost as intense as the plasmid control (lanes 1 to 6 and lane 8 of figure 3.14 C). In contrast, the 2.0 kb fragment of DNA that corresponded to wild type *cspA* in strain MPG490 (lane 7 of figure 3.14 C) produced a far less intense radioactive signal. Similarly sized DNA fragments that bound to *cspA* probe in samples 1, 2 and 3 corresponded to the native fragment from MPG490 (lanes 1 to 3 figure 3.14 C), also at a reduced level.

Analysis of extracts from the cells revealed that plasmids were present. (Figure 3.15 A shows the DNA following plasmid isolation from 14 of the 18 strains). In addition, the plasmid DNA was digested with *EcoR*I and showed that several of the plasmids had the same *EcoR*I restriction pattern as pNJH10, from where the *cspA-cat* fragment was originally PCR amplified, (figure 3.15 B). This indicated that pNJH10 had been introduced inadvertently into the host cells, albeit at a very low concentration relative to the *cspA-cat* fragment.

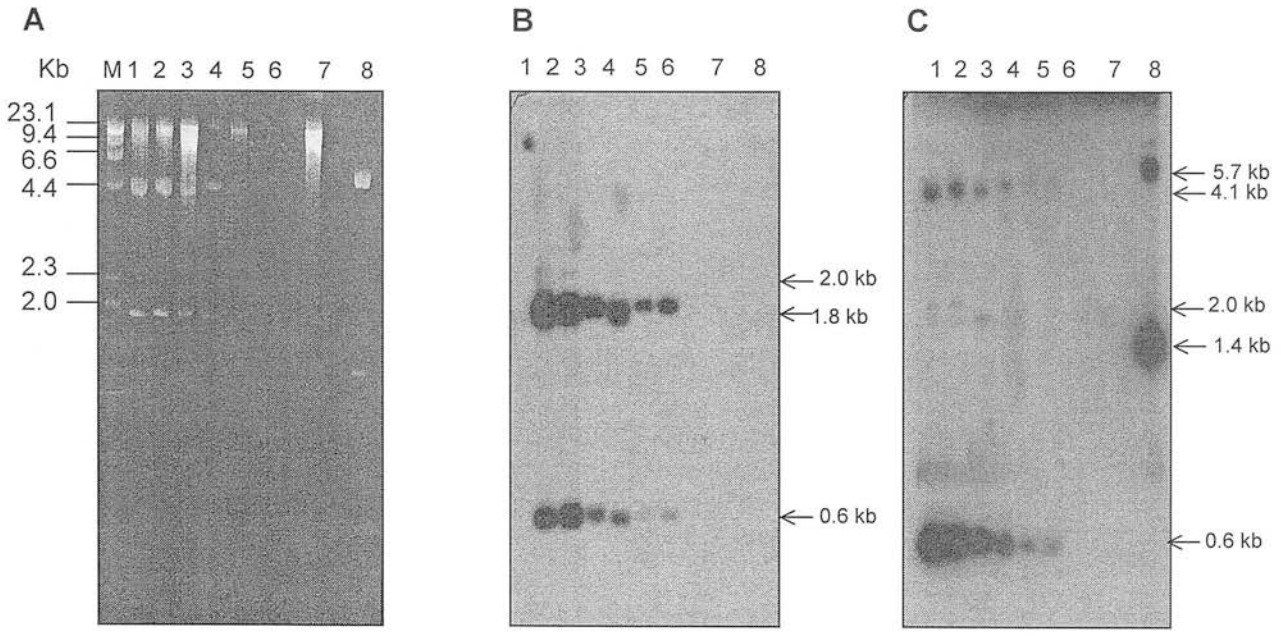


Figure 3.14 Southern blot analysis of genomic DNA from isolates of MPG490.

Analysis of chromosomal DNA from isolates of MPG490 with possible *cspA* disruptions. Southern blot showing genomic *Nco*I digests, probed with either *cat* DNA PCR amplified from pNJH10 using primers 5'-CML and 3'-CML (B) or *cspA* DNA PCR amplified from pNJH10 using primers MICG1 and MICG2 (C).
3.15 A - DNA agarose gel showing levels of DNA loaded.
3.15 B and C - corresponding autoradiographs.
Lanes 1 to 6 correspond to samples 1 to 6. Lanes 7 and 8 contain DNA from MPG490 and pNJH1, respectively and M contains the λ DNA *Hind*III ladder, with positions and sized (in kb) shown.

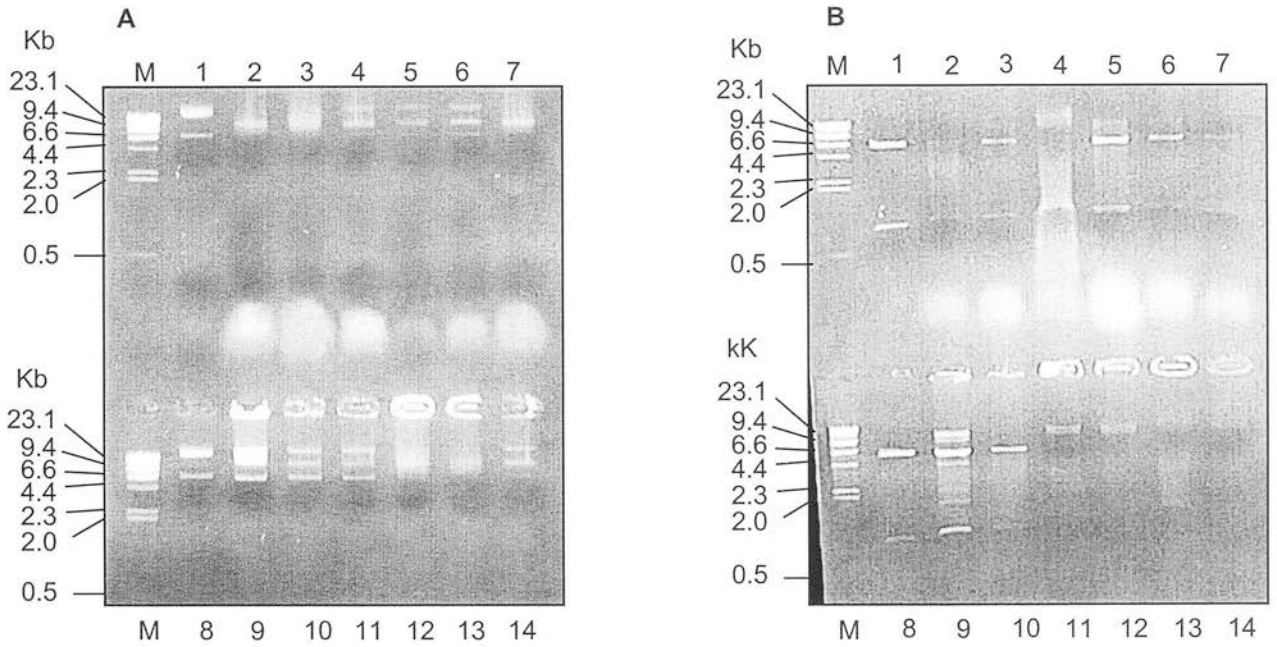


Figure 3.15 Analysis of plasmid DNA from isolates of MPG490.

Plasmid DNA prepared from isolates of MPG490 with possible *cspA* disruptions. A: uncut plasmid DNA. B: plasmid DNA digested with *EcoR*I.

3.16 A - Lanes 1 and 8 (pNJH10); lane 2 (sample 11); lane 3 (sample 18); lane 4 (sample 3); lane 5 (sample 7); lane 6 (sample 2); lane 7 (sample 10); lane 9 (sample 5); lane 10 (sample 17); lane 11 (sample 6); lane 12 (sample 13); lane13 (sample 14); lane 14 (sample 8)

3.16 B - Lane 1 and 8 (pNJH10); lane 2 (sample 7); lane 3 (sample 8); lane 4 (sample 18); lane 5 (sample 16); lane 6 (sample 2); lane 7 (sample 3); lane 9 (sample 5); lane 10 (sample 6); lane 11 (sample 10); lane 12 (sample 11); lane13 (sample 13); lane 14 (sample 14)

M is the λ DNA *Hind*III ladder, with positions and sizes shown. In both cases the sample numbers correspond to samples described in the text.

3.2.5.iv Strategy 3 - Allelic exchange using a novel method of bacteriophage co-transduction.

As an alternative strategy for recombination, a method was utilised that involved transfer of DNA via bacteriophage P22 transduction. In summary, the DNA containing the fragment to be recombined into the chromosome was cloned into a high-copy number plasmid. The plasmid was transferred into a host that contained a selectable marker, in a chromosomal locus near the gene that was to be disrupted. The presence of many copies of the plasmid in the host increased the opportunity for recombination between homologous regions of DNA on the plasmid and the chromosome. The bacteriophage was then used to transfer the fragment of DNA containing the nearby selectable marker, by transduction, to a new host. Since P22 is known to transduce approximately 1 genetic minute of chromosomal DNA (approximately 45 kb) from one host to another, DNA that was located close to the selectable marker should also be transduced to the new host. The new strain, with the disrupted gene of interest could then be cured of any residual P22 bacteriophage.

To disrupt *cspA* using this method, the first step was to sub-clone the *cspA-cat* fragment into a high copy number vector. The *cspA-cat* DNA fragment was amplified by PCR from pNJH10 using primers MICG1 and CSPA6, as described previously. The *cspA-cat* fragment was then digested with *Sal*I and inserted into the *Sal*I site of pUC19, resulting in pNJH12 (figure 3.16 A).

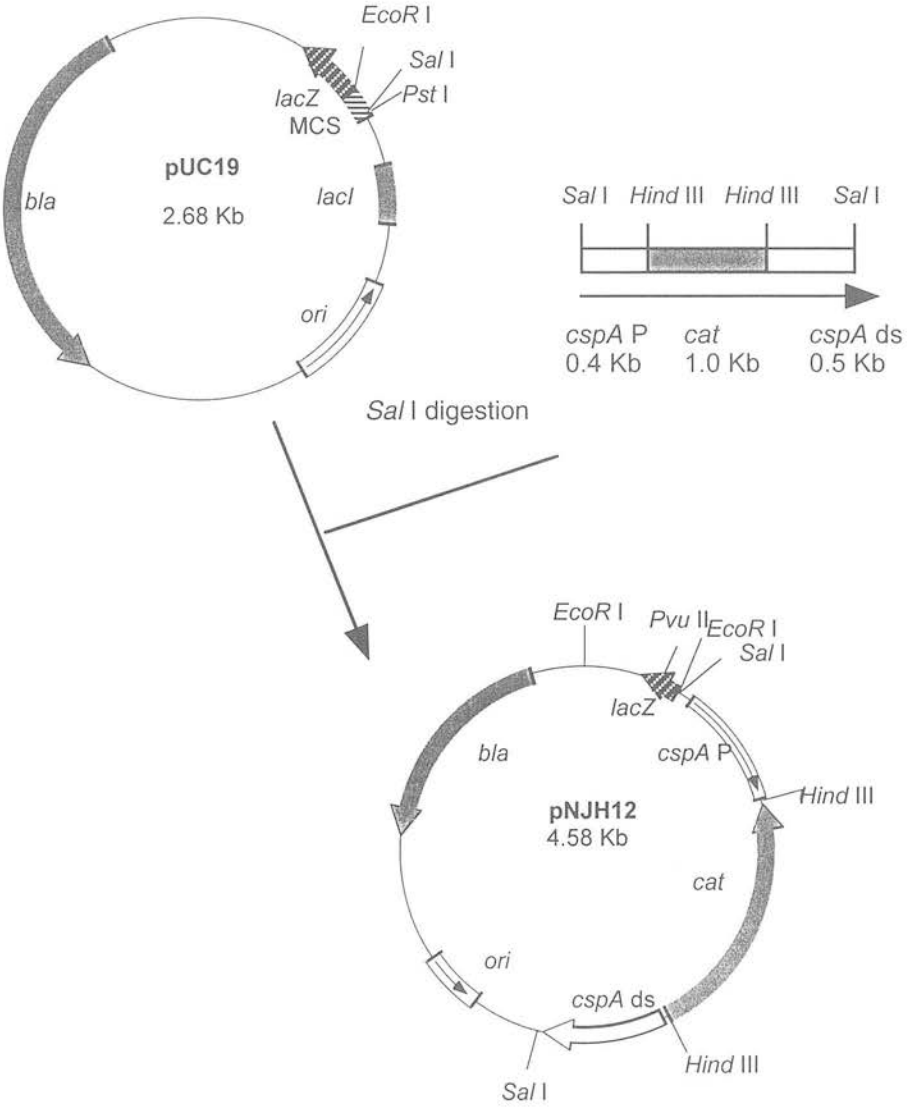


Figure 3.16 A Construction of pNJH12.

A 1.9 Kb fragment containing the *cspA* P - *cat* - *cspA ds* region was amplified by PCR from pNJH10, using primers MICG1 and pNJH6, which incorporated a*Sal* I site at the 5' end. The fragment was cloned into the corresponding site in pUC19, forming pNJH12.

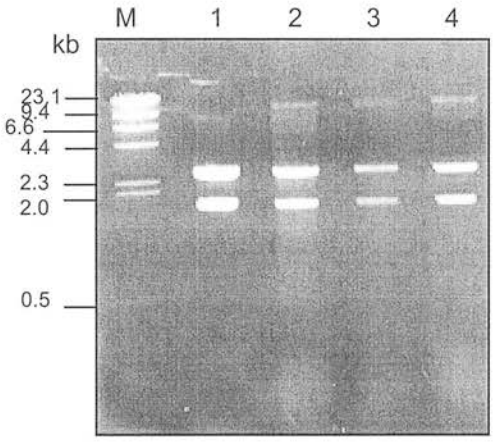


Figure 3.16 B Restriction analysis of pNJH12.

Lane 1, pNJH12 digested with *Sal*I; lane 2, pNJH12 isolated from MPG491 and digested with *Sal*I; lane 3, pNJH12 isolated from MPG492 and digested with *Sal*I; lane 4, pNJH12 isolated from MPG493 and digested with *Sal*I; M, DNA ladder (λ DNA digested with *Hind*III), the sizes and positions have been indicated.

Insertion of the *cspA-cat* fragment was confirmed by selecting for chloramphenicol resistant transformants and restriction mapping of the plasmid DNA after isolation. Digestion of pNJH12 with *Sal*I generated 2 DNA fragments of 1.9 kb and 2.7 kb, which corresponded to the *cspA-cat* fragment and the remainder of the vector, respectively (figure 3.16 B, lane 1). pNJH12 was transformed into SL1344 by electroporation, in order to change the methylation pattern, since it had been constructed in *E. coli* DH5α. The 3 strains that contained selectable markers close to the *cspA* gene were AK3312 (*pyrE27oxrE11*::Tn10), AK3040 (*xylA26*::Tn10) and AK3294 (*gltC*::Tn10). The genetic positions of the insertions are 79 minutes, 78 minutes and 80 minutes, respectively and in each case the transposon contained the *tet* resistance gene.

The selectable markers from AK3312, AK3040 and AK3294 were transferred, by P22 transduction, into SL1344 to provide a consistent genetic background and renamed as MPG491, MPG492 and MPG493, respectively. Once the strains were cured of any residual P22, pNJH12 was then transformed into each of MPG491, MPG492 and MPG493, by electroporation. Presence of the plasmid in each of the strains was confirmed by chloramphenicol selection followed by plasmid isolation and restriction with *Sal*I (figure 3.16 B). Several attempts were made to co-transduce the disrupted *cspA* gene along with the linked gene selecting on agar containing both chloramphenicol and tetracycline.

Transformation of pNJH12 into each of the host strains resulted in recovery of approximately 1500 colonies, after plating on LB agar that contained tetracycline and chloramphenicol. Approximately 20 % of these colonies were found to be ampicillin sensitive, which indicated that integration of the *cspA-cat* fragment had occurred. P22 lysates were generated from these strains and transduced into SL1344. The titre of the P22 lysates was found to range from 5×10^6 to 3×10^8 p.u.f. ml^{-1}. The transducants were grown on agar containing both chloramphenicol and tetracycline. In addition, colonies were grown on agar containing either chloramphenicol or tetracycline. Although several hundred colonies were frequently recovered in the

presence of chloramphenicol, very few were recovered on agar containing tetracycline (approximately 5 to 15 colonies per plate). However, only 1 colony derived from MPG491 was recovered that was Cml^R, Tet^R and Amp^S. Chromosomal DNA was isolated from this colony, digested with *EcoR*I and Southern blotted, as described before (figure 3.17 A and B). As positive controls, SL1344 and pNJH10 were also digested with *EcoR*I and Southern blotted. The DNA was probed with ^{32}P-labelled *cspA-cat* DNA that had been amplified by PCR from pNJH10 using primers MICG1 and CSPA6. The probe bound to the expected *EcoR*I fragments of 4.0 Kb and 900 bp in both the controls and the sample. However, as observed previously, the 900 bp fragment was evidently far more abundant than the 4 Kb fragment. Again, it was possible to isolate plasmid DNA and this generated 2 fragments following *EcoR*I digestion (figure 3.17 C). The plasmid was observed to be truncated by approximately 1 Kb relative to pNJH12 and was no longer Amp^R.

In parallel, we used the same strategy of co-transduction to recombine a disrupted copy of the *ahp* gene onto the *S. typhimurium* chromosome. The *ahp* locus has been successfully disrupted in a previous study in our laboratory and therefore served as a control for this novel method of recombination. Successful disruption of *ahp* in this manner whilst at the same time observing unsuccessful disruption of *cspA*, might indicate that *cspA* was essential for survival.

The *ahp* gene was previously disrupted using a *cat* cassette (Taylor, *et al.*, 1998). An *ahp-cat* fragment was PCR amplified from pPDT11 using primers G7858 (corresponding to bases 198-214 of *ahp*) and AHP3 (complementary and anti-parallel to bases 2069 –2093of *ahp*). These primers incorporated artificial *Bam*HI or *Nde*I restriction sites at their 5' ends, respectively. The amplified fragment was cloned into the *Bam*HI and *Nde*I sites in pUC19 and the resulting plasmid, pAHP, was transformed into SL1344 by electroporation in order to change the methylation pattern.

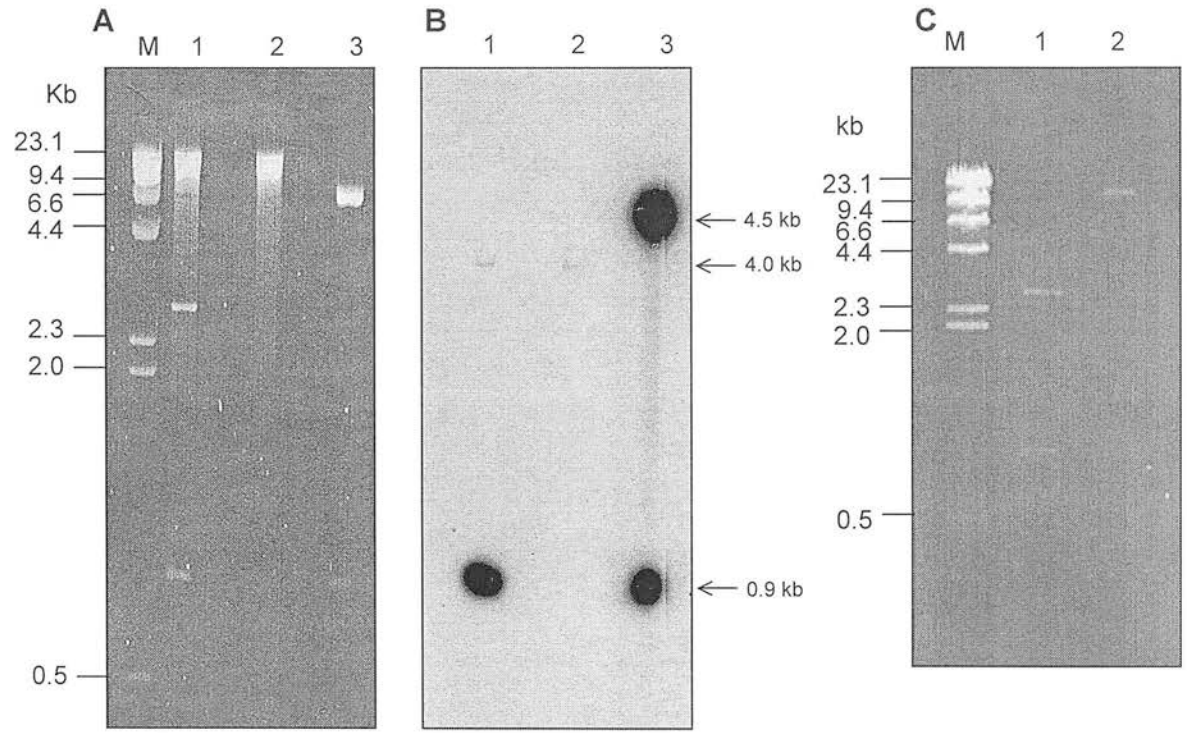


Figure 3.17 DNA analysis of an isolate from MPG491.

Analysis of DNA from isolate 12 of MPG491, with possible *cspA* disruption. Southern blot showing genomic *EcoR*I digests, probed with a *cspA-cat* fragment which was PCR amplified from pNJH10 using primers MICG1 and CSPA6.
3.18 A - DNA agarose gel showing levels of DNA loaded.
3.18 B - corresponding autoradiograph. 3.18 A and B - lane 1 (isolate 12); lane 2 (SL1344); lane 3 (pNJH10).
3.18 C - plasmid DNA isolation from isolate 12. Lane 1 (isolate 12 digested with *EcoR*I); lane 2 (uncut plasmid DNA from isolate 12); M contains the λ DNA *Hind*III ladder, with sizes and positions shown in panel A.

The selectable markers within close proximity to *ahp* (which is located at 14.2 genetic minutes on the chromosome), from TN1744 and TT7247, were transferred into a SL1344 background, to maintain a consistent genetic background and renamed MPH494 and MPG495, respectively. Plasmid pAHP was transformed into these strains by electroporation and several attempts at co-transduction were carried out. The colonies were plated on LB agar plates with appropriate antibiotics to give an indication of the transduction efficiency. The plates contained either chloramphenicol and tetracycline, chloramphenicol only, or tetracycline only. Although several hundred Cml^R colonies were recovered following transduction from both MPG494 and MPG495 and 8 Tet^R colonies were derived from MPG495, no co-transductants were recovered that were Cml^R and Tet^R. These results indicated that this method was not universally successful for allelic recombination, although it was possible that in the case of MPG495, the selectable markers were not sufficiently close to the *ahp* locus to be co-transduced with a high frequency.

3.3 Discussion

Shifting cultures of many bacteria to low temperatures results in induction of an adaptive mechanism, termed the cold shock response. A major feature of the response is the induction of cold shock proteins. The major cold shock proteins, CspA, of *E. coli*, and CspB of *B. subtilis*, have been shown to be involved in regulating low temperature induction of proteins and *E. coli* CspA has been reported to act as an RNA chaperone (Bae *et al.*, 1997; Graumann *et al.*, 1997; Jiang *et al.*, 1997). Furthermore, the presence of cold-inducible CspA homologues has been demonstrated in a wide range of bacteria and database searches of recently sequenced bacterial genomes continue to reveal the presence of new CspA homologues. The *S. typhimurium cspA* gene is highly similar to *E. coli cspA*, to the extent that the derived amino acid sequences are identical. Consequently many characteristics that have been found in *E. coli cspA* may be applicable for *S. typhimurium cspA*. In this study, attempts were made to disrupt *S. typhimurium cspA*, on the chromosome, by allelic exchange.

Various studies have been carried out to determine the role of the major cold shock protein during the cold shock response. These studies have focused on *E. coli cspA* and *B. subtilis cspB*. The amino acid sequences of these proteins bear a high level of similarity to eukaryotic Y-box proteins. For example, *E. coli* CspA shares 43 % amino acid identity to the so-called cold shock domain of the eukaryotic Y-box proteins. Y-box factors, such as human YB1 and *Xenopus* FRG Y2, are a family of DNA-binding proteins that bind to the Y-box motif, which has a consensus sequence of CTGATTGG$^{C}/_{T}{}^{C}/_{T}$AA (Lee *et al.*, 1994). Similar functions to those exhibited by eukaryotic Y-box factors have been explored for the bacterial major cold shock proteins, and several studies have shown that *E. coli cspA* and *B. subtilis cspB* both bind to DNA that contains part of the Y-box motif (ATTGG), although with differing affinities. *In vitro* studies with *E. coli* have shown that purified CspA binds to *E. coli gyrA* duplex DNA that contains three ATTGG motifs. Sequential mutation of regions of DNA that contained the motifs resulted in proportional reduction of DNA/protein complexes. Purified *E. coli* CspA has also been shown to bind to a region of the *E.*

coli hns promoter that contained a CCAAT motif (Brandi *et al.*, 1994). The affinity of CspA for this region of DNA was lower when the protein was in purified form as opposed to extracts from cold shocked cells (following incubation for 3 hours at 10°C). In each case, a 2-fold molar excess of CspA resulted in formation of a DNA/protein complex (Jones *et al.*, 1992). This suggested that some other factor, present in the cell extracts was responsible for the differences in affinity. The authors suggested that this was RNA polymerase.

Nucleic acid binding studies have shown that the major cold shock protein of *B. subtilis*, CspB, displays a similar ability to bind DNA to that of CspA of *E. coli*. For example, a 2-fold molar excess of CspB resulted in formation of a DNA/protein complex with ssDNA containing the ATTGG motif. The protein also bound to ssDNA containing the complementary motif, although the binding affinity was reduced by 50% (Graumann *et al.*, 1994). CspB did not bind to duplex DNA derived from the ssDNA.

These studies show that the major cold shock proteins of both *E. coli* and *B. subtilis* may act as DNA-binding proteins *in vivo*, and may therefore act in a similar fashion to the eukaryotic Y-box factors. Interestingly, the synthesis of both *E. coli* GyrA and H-NS has been demonstrated to increase, between 2 and 10-fold, following a shift from 37°C to 15°C (Jones *et al.*, 1992). Thus, it has been speculated that *E. coli* CspA may play an important role in the cold shock induction of these proteins. Furthermore, many *cspA* homologues contain the ATTGG motif, or degenerate version of it, near their promoter sequences. However, since degeneracy of just 1 base increases the frequency of this sequence being present, the proposal that CspA homologues may utilise this sequence as a target for auto-regulation has been met with a degree of scepticism.

A further characteristic of the Y-box motif proteins is the presence of RNA binding motifs, RNP1 and RNP2. These motifs are also present in a highly conserved region of CspA homologues, although only the partial RNP2 motif is present. These motifs

are normally present on the 3rd and 1st β strands, respectively, of the eukaryotic proteins (for a review see Burd & Dreyfuss, 1994). The protein structures of *E. coli* CspA and *B. subtilis* CspB have been elucidated and show that the proteins form 5 stranded β-barrels, with the RNP motifs present on the 1st and 3rd β strand, respectively (Schindelin *et al.*, 1994). Studies with *B. subtilis* have shown that CspB could bind to RNA that was derived from DNA containing the ATTGG motif, although the affinity was 2-fold lower than for the equivalent ssDNA molecule. Another *B. subtilis* cold inducible protein, CspD, also bound to this RNA, although its binding affinity was almost 2-fold lower than for CspB (Graumann *et al.*, 1997). Investigation of the RNA binding affinities of *E. coli* CspA have shown that this protein has a similar affinity for RNA as for ssDNA, so that in both situations the concentration of CspA that was required to form a nucleic acid/protein complex was approximately 2.7×10^{-5} M (Jiang *et al.*, 1997). These *in vitro* studies show that members of the CspA family can bind RNA. However, as yet, their role as transcriptional regulators has not been proven conclusively

It has been suggested that *E. coli* CspA may also act as an RNA chaperone (Jiang *et al.*, 1997). Addition of purified CspA to mRNA (derived from the 5'-UTR of *E. coli cspA*) led to enhanced degradation of the transcript in the presence of RNaseA and RNase T1. This activity appeared to be co-operative, since the concentration of CspA required for the degradation was considered to be high (2.7×10^{-5} M) relative to the estimated cellular level.

The role of the *B. subtilis* CspB has been investigated in low temperature survival. Chromosomal disruption of the *cspB* gene did not alter cell survival at low temperature. However, disruption of *cspB* combined with mutation of 2 other cold inducible CspA homologues led to the absolute requirement of complementation of the triple mutant strain with *cspB* on a plasmid at 37°C and 10°C. Thus, the combination of the cold shock proteins CspB, CspC and CspD were essential for survival at optimal and low temperatures (Graumann *et al.*, 1997). To date, this study stands alone in demonstrating the essential nature of the cold shock protein, under

certain conditions. In contrast, deletion of *E. coli cspA* did not affect cell growth or survival at low temperatures, although synthesis of some of the cold inducible proteins was affected (Bae *et al.*, 1998) It is possible that simultaneous deletion of the other cold inducible CspA homologues in *E. coli* may lead to a similar finding as for *B. subtilis*.

This chapter reports on several attempts to disrupt the chromosomal copy of the *S. typhimurium cspA* gene. Unfortunately, none of these attempts resulted in a double homologous cross-over event. However, one attempt led to a number of recombinant strains in which single homologous cross-over events occurred (figure 3.7). In these strains, *S. typhimurium cspA* was disrupted by insertion of *lacZ* at base 376, and a *cat* cassette was included downstream of *lacZ* for antibiotic selection. Analysis of the chromosomal DNA from these strains indicated that the native copy of *cspA* was still present in addition to the disrupted copy of *cspA*. Restriction analysis of the region downstream of *cspA* indicated that the *cspA-lacZ-cat* fragment had been inserted downstream of the native, chromosomal copy of *cspA*. It is possible that the double cross-over event did not occur due to the dis-proportionally long 3' region downstream of *cspA* and the presence on the construct of a large non-homologous 4.1 Kb insert. This problem was addressed by further attempts at allelic exchange that involved a shorter truncation of the *cspA* ds region.

A *cspA-cat* construct was designed where only the *cat* cassette was inserted at base 376 of *cspA*, and the *cspA* ds region was truncated to 500 bp, which was similar to the length of the *cspA* P region. Homologous recombination was attempted in a *recD* (Russell *et al.*, 1989) host. The *recD* exonuclease is one of several nucleases responsible for degradation of linear DNA. Thus, mutations of *recD* result in robust and recombination proficient mutants that tolerate greater concentration of linear DNA within the cells and this led to a strategy that involved transformation of the strain with linearised DNA, rather than circular DNA. The linearised DNA (containing *cspA-cat*) was amplified by PCR from pNJH10 and introduced into the host cells by electroporation. Several colonies were recovered where the *cspA-cat*

construct appeared to have successfully integrated onto the chromosome. However, further analysis showed that the multicopy plasmid, pNJH10, from which the *cspA-cat* construct was derived, was present in the strains. It is most likely that the plasmid (pNJH10) was inadvertently introduced into the cells together with the linearised DNA. This occurred because the *cspA-cat* PCR product was not isolated from the plasmid template following amplification by PCR, and would have most likely been avoided if alternative purification steps, such as agarose gel purification, had been taken. On this occasion, the transformation efficiency of the cells was tested by electroporation of pNJH10. (Transformation of 100 ng of plasmid into 1 x 10^9 cells resulted in recovery of approximately 5000 c.f.u. ml^{-1} on selective LB agar plates).

As an alternative method, co-transduction by *S. typhimurium*-specific P22 was attempted (adapted from N.F. McLennan & M. Masters, unpublished data). This novel method used a strain that carried a Tet selectable marker present on a Tn*10* in close genetic proximity to the *cspA* locus. A high copy number plasmid, carrying the *cspA-cat* fragment, was transformed by electroporation into the strain bearing the selectable marker (the *tet* gene). It was expected that following transduction, selection of this marker together with selection of the *cat* marker would enrich for isolation of recombinants where exchange had occurred. The strain was also tested for sensitivity to ampicillin, since the plasmid vector contained *bla*. It was found to be sensitive to ampicillin. However, DNA analysis showed that plasmid DNA was still present in the strain, although on this occasion, the plasmid was truncated relative to the original vector and had lost resistance to ampicillin. To our knowledge, this method had only been used previously in *E. coli* bacteria, using P1 transduction, and had not been tested with *S. typhimurium*.

Together these results show that disruption of *S. typhimurium cspA* is not straight forward and suggests that *cspA* may be essential for cell survival, even at optimal temperatures (i.e. 37°C). The *cspA* gene of *E. coli* was chromosomally disrupted by Bae & colleagues (1997) using a positive and negative selection method developed by this group. In this case a *cspA-cat* fusion was constructed on a plasmid, containing 620 bp of the region upstream of *cspA* and 2.2 Kb of the region downstream of *cspA*

and the *cspA* ORF was replaced with the *cat* gene. The plasmid carried the *E. coli* $galK^+$ gene and a temperature-sensitive origin of replication, which meant that replication occurred only at a permissive temperature (30°C). Thus, transformation at 42°C in $galK^-$ host cells led to co-integration. Recombinants were recovered in the presence of chloramphenicol and 2-Deoxygalactose, which allowed the disruptants to be isolated from the co-integrants. This construct bears several similarities to the one that was used for allelic recombination attempts in *S. typhimurium*. In particular, it is interesting that allelic recombination of *E. coli cspA* was successful when the region downstream of *cspA* was dis-proportionally long in comparison to the region upstream of *cspA*. In this chapter, it was thought that such a strategy would lead to an increase in the proportion of single cross-over events occurring, thereby, reducing the proportion of double cross-over recombination events. However, the procedure described for disruption of *E. coli cspA* used a counter selection method to isolate disruptants from co-integrants. Thus, this method 'forced' recombination and resulted in isolation of cells that contained a disrupted copy of *cspA*.

In contrast, the methods used by Graumann & colleagues (1997), to disrupt *B. subtilis cspB*, *cspC* and *cspD*, were relatively straight forward. In each case, the upstream and downstream regions flanking the *csp* genes were PCR amplified and cloned into pBluescriptII KS. DNA containing either the *cml* or *kan* genes was then inserted in between the *csp* flanking regions. Subsequently, host *B. subtilis* cells were transformed with the plasmids that do not replicate in *B. subtilis* and recombinants were positively selected on agar containing the appropriate antibiotic. In addition, double *csp* mutants were constructed by transforming host $cspB^-$ (*cspB::cat::spec*) cells with chromosomal DNA from $cspC^-$ (*cspC::kan*) and $cspD^-$ (*cspD::cat*) strains, respectively and similarly transforming $cspC^-$ (*cspC::kan*) $^-$ with chromosomal DNA from $cspD^-$ (*cspD::cat*). The efficiency of mutation of the double mutants varied, so that approximately 100 transformants that showed deletion of both *cspC* and *cspD* were recovered, compared to only 2 transformants that were deleted for both *cspB* and *cspC*. However, attempts to create a triple *csp* mutant, using similar procedures to those described previously, were unsuccessful. The $cspC^-$ $cspD^-$ strain was

subsequently transformed with a plasmid that carried the *cspB*$^{+}$ gene under the control of an IPTG-inducible promoter. By this route the chromosomal *B. subtilis cspB* gene could be disrupted and was shown to be essential in the *cspC*$^{-}$ *cspD*$^{-}$ background at optimal and sub-optimal temperatures. Neither *E. coli cspA* nor *B. subtilis cspB* single deletions were found to affect cell survival adversely (Bae *et al.*, 1997; Graumann *et al.*, 1997).

These studies show that although disruption of *csp* genes is possible in some instances, extreme methods may be required. The major *csp* gene is considered to be essential in *B. subtilis* cells if the other cold inducible *csp* genes are disrupted. This supports the findings in this study that chromosomal disruption of *S. typhimurium cspA* was not straightforward. In contrast, the *cspB* gene of *S. typhimurium*, which is cold inducible, has been successfully disrupted in our laboratory previously (Craig *et al.*, 1998), although, 23 codons of the *cspB* ORF remained intact in the disruption. Proteome analysis with this *cspB*$^{-}$ strain has shown that CspA is de-repressed after incubation for 4 hours at 10°C (see chapter 6 of this thesis). The findings support similar studies with the *E. coli cspA*$^{-}$ strain, where production of CspB and CspG increased after 4 hours at 10°C, relative to the wild type strain (Bae *et al.*, 1997). The levels of de-repression of *S. typhimurium* CspA appeared to be slightly higher than *E. coli* CspB and CspG.

In order to gain a fuller understanding of the role of the major cold shock protein in *S. typhimurium*, disruption of *cspA* remains a priority. However, it is also necessary to uncover and disrupt any hither-to unknown cold inducible *csp* genes, since their presence may compensate for the loss of *cspA*. The use of proteomics together with very sensitive identification techniques provide valuable tools in the detection and identification of proteins that are induced in response to stresses such as cold shock. Some of these techniques were used to characterise the cold shock response of *S. typhimurium* (chapter 6 of this thesis). The use of proteomics together with sensitive protein identification techniques is increasingly common. For example, Spector and colleagues have made extensive use of proteomics to determine which proteins were

induced following starvation, anaerobic shock and heat shock (Spector *et al.*, 1986). In addition, DNA chip technology is also emerging as a potentially valuable tool for characterisation of genes involved in stress responses (Richmond *et al.*, 1999). Once identified using such techniques, mutation of cold inducible genes would allow phenotypic characterisation.

In this chapter, DNA downstream of the *S. typhimurium cspA* gene was sequenced so that a oligonucleotide primer could be designed for disruption studies. The sequence revealed a putative ORF, in which the putative amino acid sequence exhibited 24.5% identity over a region of 139 amino acids to a subunit of an alkylmercury lyase from *Staphylococcus aureus* (Laddaga *et al.*, 1987). Several *mer* genes are involved in either narrow spectrum resistance to inorganic mercury, or broad spectrum resistance to organomercurials. The *merB* gene product encodes an enzyme that is involved in the latter resistance mechanism. Although it is tempting to speculate that such environmental resistance genes may be present downstream of the cold shock genes in *S. typhimurium*, and that there may even be some form of co-expression following exposure to environmental stresses, there are factors which suggest that this may not be so. The level of amino acid identity is relatively low and furthermore, mechanistically important cysteine residues that are conserved amongst MerB proteins from different organisms are not conserved in the *S. typhimurium* ORF (Kiyono *et al.*, 1997). The mercury resistance genes are commonly plasmid based rather than chromosomally encoded, although they are often found in transposable elements.

The role of the major cold shock protein has still to be fully elucidated although *in vitro* evidence suggests that *E. coli* CspA and *B. subtilis* CspB are DNA or RNA binding proteins involved in adapting the cell to function at low temperatures. It is clear that these proteins play a key role in cold shock adaptation. However, the majority of bacteria that have CspA-type proteins contain several homologues. It has been suggested that CspA homologues have arisen as a result of gene duplication, in which case the functions of some of the proteins are likely to overlap. The small

number of bacteria identified so far, that lack CspA-like proteins, tend to be host-adapted. For example, *Helicobacter pylori* is especially well adapted to live in the gut and is one of the most common causes of chromic gastric diseases in humans, while *Mycoplasma genatalium* is a parasite of a wide range of hosts including primates (Tomb *et al.*, 1997; Fraser *et al.*, 1995). It is possible that they do not require CspA-like proteins because they do not encounter environmental variations that bacteria, such as *Salmonella* and *Escherichia*, which have a more complex lifestyle, encounter.

CHAPTER 4

EXPRESSION OF *S. typhimurium cspA* AND *cspB* AT LOW TEMPERATURES, AND THE EFFECT OF GLOBAL REGULATORS DURING THE COLD SHOCK RESPONSE.

4.1 INTRODUCTION

The cold shock response is similar to other stress responses such as heat shock. A universal stress response can be defined by the following; firstly a rapid, transient change in cellular activities which increases survival during periods of stress, secondly, essential components are protected against damage, and thirdly, normal cellular activities are resumed during the recovery period (Burton, 1986). A reduction in the ambient temperature to sub-optimal levels results in the cold shock response, with enhanced ability of the bacteria to survive exposure to lower temperatures. *E. coli*, a mesophile like *S. typhimurium*, has been shown to respond to incubation at low temperature with transient induction of a specific set of cold shock proteins and repression of the majority of proteins, including heat shock proteins (Jones *et al.*, 1987). In some bacteria, there is a lag in growth following a temperature reduction, e.g. in *S. enteritidis* there is a lag of 6 hours following a shift from 37°C to 10°C. During this period the cold induced proteins are synthesised (Jeffereys *et al.*, 1998, Jones, *et al.* 1987). Two-dimensional electrophoresis has shown that CspA, the major cold shock protein, is the most highly induced protein following cold shock in many bacteria, including *S. typhimurium* (this thesis, chapter 6). The exact role of CspA is still unknown, although it is appears to be involved in adapting the bacterial cell to the lower temperature. CspA belongs to a family of highly related proteins, some of which are also cold shock inducible. Examples include CspA and CspB in *S. typhimurium* (this thesis, chapter 6, Craig *et al.*, 1998), CspA, CspB, CspG and CspI of *E. coli* (Lee *et al.*, 1994, Nakashima *et al.*, 1996) and CspB, CspC and CspD of *B. subtilis* (Graumann *et al.*, 1996).

4.1.1 Regulation of expression of *cspA* homologues.

Regulation of the cold shock proteins has been most extensively studied in *E. coli*, in particular of CspA. Many factors appear to be involved at the levels of transcription, mRNA stability and translation. At the time of writing, the stability of the transcript appears to be especially important. Interestingly, unlike other stress responses,

including the heat shock responses, an alternative sigma factor has not been reported in regulation of the cold shock response. Studies have indicated that distinct differences occur in the thermoregulation of *E. coli cspA* and *cspB* genes (Etchegaray *et al.*, 1996). In addition to the frequently reported induction of the CspA protein following shifts of exponentially growing cells from 37°C to 15°C or 10°C, CspA was also detected following shifts from 37°C to 24°C and from 42°C (which is above the optimal growth range for *E. coli*) to 29°C (Jones *et al.*, 1992). Thus it seems that it is the degree of the temperature downshift that induces CspA expression, rather than activation of expression below a defined temperature threshold. In contrast, studies with *E. coli* CspB have shown that induction of this protein occurs following a shift from 37°C to a temperature between 20°C and 10°C, suggesting that a temperature threshold may operate for activation of *cspB* expression (Etchegaray *et al.*, 1996).

The following section reviews what has been shown in terms of regulatory controls for CspA expression, in order of significance, with the most important factor first.

4.1.1.i The role of mRNA stability.

Several studies have shown that differential stability of the mRNA of the genes encoding cold inducible CspA-like proteins appears to play a major role in their regulation. In general, the transcripts are very unstable around the optimal growth temperature, e.g. 37°C for *E. coli*, but become far more stable following a temperature downshift. Studies with *E. coli* have shown that the half life of *cspA* mRNA was less than 15 seconds at 37°C, for a culture that was grown to mid-exponential phase at 37°C. In contrast the half life increased to more than 60 minutes, when the culture had been continuously grown at 15°C for 20 hours (Goldenberg *et al.*, 1996). In addition, this group also examined the RNA stability of two separate *cspA* hybrids. A transcriptional *cspA::lacZ* fusion was constructed, where the *lacZ* gene was fused to *cspA* at position +81 (upstream of the ribosome binding site), and a translational *cspA::lacZ* fusion was also constructed, where *lacZ* was fused within the ORF of *cspA* at position +346. The *cspA-lacZ* fusions were integrated onto the

chromosome at the λ attachment site forming a heterodiploid and thus allowing simultaneous analysis of both native *cspA* and *cspA-lacZ* mRNA species. In the case of the transcriptional fusion, the mRNA half life increased dramatically to approximately 90 minutes, when the culture was exponentially growing at 37°C. Furthermore, when the culture was grown exponentially at 37°C and shifted to 15°C, the half life of the mRNA increased to more than 60 minutes. This was independent of the time of sampling after the temperature shift, i.e. at 30, 60 or 120 minutes. When the cells were grown continuously at 15°C for 20 hours, the half life of the fusion was approximately 15 minutes. Similar results showing an increase in stability were also reported from studies of the RNA transcript for the translational *cspA::lacZ* fusion. This indicated that stabilisation of the hybrid transcript was either due to the *lacZ* portion of the RNA hybrid, or the removal of a signal for mRNA degradation by construction of the fusions. In order to judge the effect of a temperature shift on a non-cold shock inducible gene, the same experiments were carried out on the leader sequence of the *rrn* ribosomal RNA. The RNA half life of *rrn* was approximately 10 minutes at 37°C and 15 minutes at 15°C and The stability of the *rrn* transcript was not affected by the length of incubation at 15°C (Goldenberg *et al.*, 1996).

The stability of *E. coli cspA* and *cspB* mRNA has also been studied over a range of temperatures. The expression of *cspA* mRNA was found to be uniformly high following temperature down-shifts from 37°C to 20°C, 15°C or 10°C and was only marginally reduced at 6°C. For each temperature, mRNA was isolated after 20, 40 or 60 minutes, and *cspA* mRNA was found to peak after 40 minutes at each temperature. Similarly, CspA protein was induced to relatively high levels over a range of temperatures; following shifts from 37°C to 24°C, 20°C and 15°C for 30 minutes in each case. On the other hand, *cspB* mRNA, isolated in the same manner as *cspA* mRNA, was most highly produced following a shift from 37°C to 15°C and production peaked after 20 minutes at this temperature. Moreover, *cspB* mRNA was barely detectable at 30°C, 24°C or 6°C, which suggests that *E. coli cspB* is induced following a shift to a threshold temperature, around 20°C. CspB protein induction was also considerably more temperature-dependent than CspA protein induction. The

highest level of CspB expression was observed at 15°C, and decreased to 40% of the peak level at 20°C and 30% at 10°C. (For each temperature and for both CspA and CspB, protein labelling with ^{35}S-methionine took place after incubation for 30 minutes, with the exception of 6°C, where incubation was extended to 2 hours before protein labelling) (Etchegaray *et al.*, 1996).

The presence of a threshold temperature for cold shock induction has also been demonstrated for *S. typhimurium cspB* by Northern analysis and using a bioluminescent reporter system (Mu*dlux*) that was fused within the ORF of *cspB* (at position +223; Craig *et al.*, 1998). The latter study found that *cspB* mRNA was detected following a shift from 30°C to 22°C and 10°C, but not at 24°C. Studies of *cspB* mRNA stability showed that the *cspB* transcript was detectable for at least 60 minutes at 10°C in the presence of rifampicin. However, shifting the culture after 1 hour incubation at 10°C, to 24°C, in the presence of rifampicin, resulted in loss of *cspB* mRNA within 15 minutes. Similarly, bioluminescence from the *cspB::*Mu*dlux* fusion was at least 1 order of magnitude higher when the culture was shifted from 30°C to 22°C as opposed to 24°C, for 2 hours. Furthermore, when a culture was shifted from 24°C to 22°C, the level of bioluminescence increased by more than 2 orders of magnitude, after 2 hours (Craig *et al.*, 1998). Thus it appears that *S. typhimurium cspB* RNA is stabilised below a threshold temperature of 24°C and is rapidly degraded at higher temperatures (following temperature upshift from 10°) by pre-existing enzymes.

More recent studies of *E. coli cspA* have suggested that the *cspA* mRNA is constitutively produced at 37°C, but is very rapidly degraded at this temperature (Fang *et al.*, 1997). A putative RNase E recognition site has been proposed near the ribosome binding site of the *E. coli cspA* gene, and a 3 base substitution in this region resulted in partial stabilisation of *cspA* mRNA at 37°C. It is likely that this substitution altered the secondary structure of the *cspA* 5'UTR lowering its suitability as a substrate for RNase E (figure 4.1).

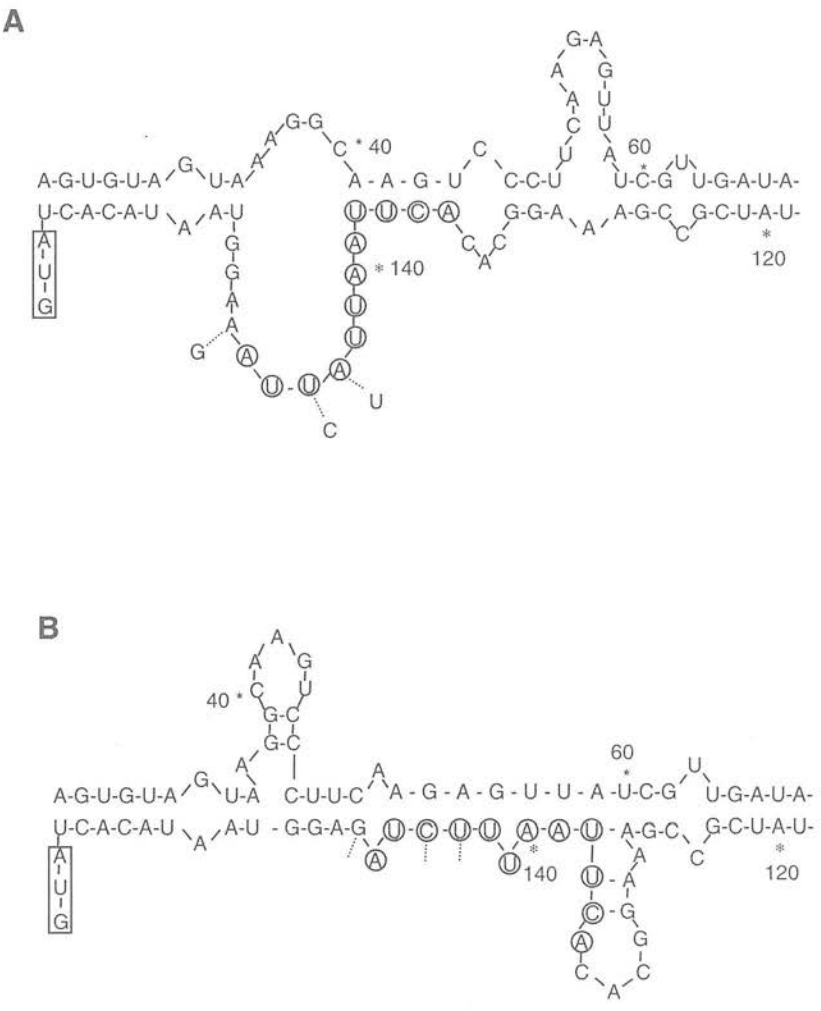


Figure 4.1 Putative RNase E degradation motif in *E. coli cspA*.

Proposed secondary structure of part of the 5'-UTR for wild type *cspA* (**A**) and the proposed structure for the mutated *cspA* mRNA with 3 base substitution (**B**). Bases in the putative 12-base RNase E-cleavage region are indicated by circles and 3-base substitutions are indicated with dotted lines. The translational start codon is boxed. (This figure has been adapted from Fang *et al.*, 1997).

Δ *cspA* strain and the parental strain, following incubation at 15°C for 30 minutes. However, following 3-hour incubation at 15°C, *cspA* mRNA half-life from the Δ *cspA* strain was approximately 2-fold higher than *cspA* mRNA from the wild type strain. The stability of the mRNA from both Δ *cspA* and the wild type *cspA*, decreased from more than 60 minutes, after incubation at 15°C for 30 minutes, to 25 minutes, after incubation at 15°C for 3 hours. CspA was also found to exert a minor regulatory effect on CspB and CspG, and both mRNA half-lives and protein levels of CspB and CspG were found to increase approximately 1.4-fold, in the Δ *cspA* strain, relative to the wild type strain (Bae *et al.*, 1997).

The presence of a 14 bp sequence, termed the downstream box (DB), located within the first 10 codons of *E. coli cspA* has been implicated as an additional regulatory element and is proposed to affect translational efficiency of *cspA* mRNA. Evidence was provided to support this hypothesis in that β-galactosidase levels were reduced by 50% for a *cspA-lacZ* fusion where *lacZ* was fused to the 4th codon of *cspA*, when compared to a similar *cspA-lacZ* fusion, where *lacZ* was fused to the 13th codon of CspA (Mitta *et al.*, 1997). Several *E. coli* and bacteriophage transcripts containing downstream box sequences have been shown to interact with a complementary sequence near the decoding region of 16S rRNA, and this is thought to mediate efficient translation from the initiation codon independently of the SD sequence (Sprengart *et al.*, 1996). The *E. coli cspA* DB contains 10 nucleotides that are complementary to the 16S rRNA anti-DB (figure 4.2).

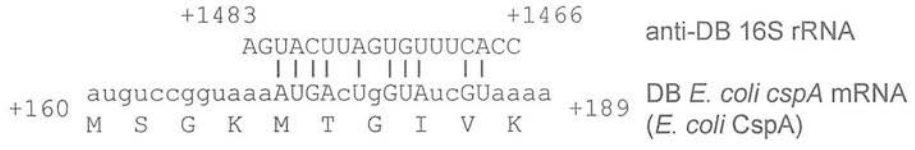


Figure 4.2 Complementarity between the putative *E. coli cspA* downstream box and 16S rRNA anti-downstream box.
The *cspA* mRNA sequence shown corresponds to the first 10 codons of the ORF. The numbers given in superscript represent the position of the gene sequence, relative to the transcriptional start positions. (Adapted from Mitta *et al*, 1997).

An additional region termed an UP element, located roughly within the 20 bp upstream of the –35 sequence of the *E. coli cspA* promoter, also appears to be important for CspA accumulation following cold shock. Deletion of the UP element resulted in a 4-fold decrease in β-galactosidase levels from a *cspA-lacZ* transcriptional fusion, following incubation at 15°C for 3 hours (Mitta *et al.*, 1997). It has been reported previously that the UP element (an AT rich region) located upstream of the –35 region of bacterial promoters, stimulates transcription by interacting with the α-subunit of RNA polymerase (Ross *et al.*, 1993). Many *csp* genes have such AT rich regions in the 5'-UTRs, including *S. typhimurium cspA* (this thesis, chapter 3) and *cspB* (Craig *et al.*, 1998).

4.1.1.iii Differential regulation of CspA homologues.

The regulation of *E. coli cspB* appears to be similar in many reports to that of *E. coli cspA*, although differences have been observed in how differential thermo-regulation appears to operate. Studies of mRNA stability and protein levels have shown that *E. coli cspB* expression occurs over a narrower temperature range than *E. coli cspA* (table 4.1).

Temperature (°C)	*E. coli* CspA	*E. coli* CspB
6	0.05	0.05
10	0.7	0.3
15	1.0	1.0
20	0.9	0.4
25	0.8	0.1
30	0.2	0.1

Table 4.1 Differential cold shock induction of *E. coli* CspA and CspB.
The cold shock induced bands, detected by 2-dimensional electrophoresis, were analysed in a phosphorimager. The level of induction is given relative to the highest level (which = 1.0) for each protein. For each temperature and for both CspA and CspB, protein labelling with ^{35}S-methionine took place after incubation for 30 minutes, with the exception of 6°C, where incubation was extended to 2 hours before protein labelling. The labelling took place over 15 minutes in each case. (Adapted from Etchegaray *et al.*, 1996).

Thus, it appears that induction of *E. coli* CspB occurs following a shift from 37°C to a range between 20°C and 10°C, whereas CspA expression appeared to occur over a wider temperature range and the degree of the temperature downshift seems more important in the induction of CspA. In a similar fashion, *S. typhimurium cspB* has been shown to be induced below a threshold temperature of approximately 22°C (Craig *et al.*, 1998). Expression of a *cspB* translational fusion was induced following a shift from 30°C to 22°C, but was only poorly produced at a basal level following a shift from 30°C to 24°C. These reports correlate well with studies of *S. typhimurium cspB* mRNA which was shown by Northern hybridisation to be produced below a specific temperature threshold and was destabilised at higher temperatures (Craig *et al.*, 1998).

4.1.2 *S. typhimurium* CspA paralogues.

S. typhimurium strain SL1344 has been shown to contain at least 2 cold shock genes, *cspA* (J. Craig & L. Chamberlain, unpublished data) and *cspB* (Craig *et al.*, 1998). Two additional *S. typhimurium cspA* homologues have also been submitted into the EMBL database. Both *S. typhimurium cspA* and *cspB* contain most of the putative regulatory elements that have been cited for *E. coli cspA* homologues. These elements are indicated in figure 4.3 of this thesis for *cspA* and figure 4.4 for *cspB*. The amino acid sequence of CspB and CspA share 64% identity. Both genes contain the AT-rich putative, regulatory region upstream of the –35 site. In *cspB*, this is around –40 to –60, which is similar in the location to *cspA*. A repressor element, termed the cold box, is present in some cold shock genes, such as *E. coli cspA, cspB* and *csdA*. Fang *et al.* (1998) have shown by overexpressing the 5'-UTR containing the cold box of *E. coli cspA* and *cspB* that *E. coli* CspA, CspB and CsdA are derepressed.

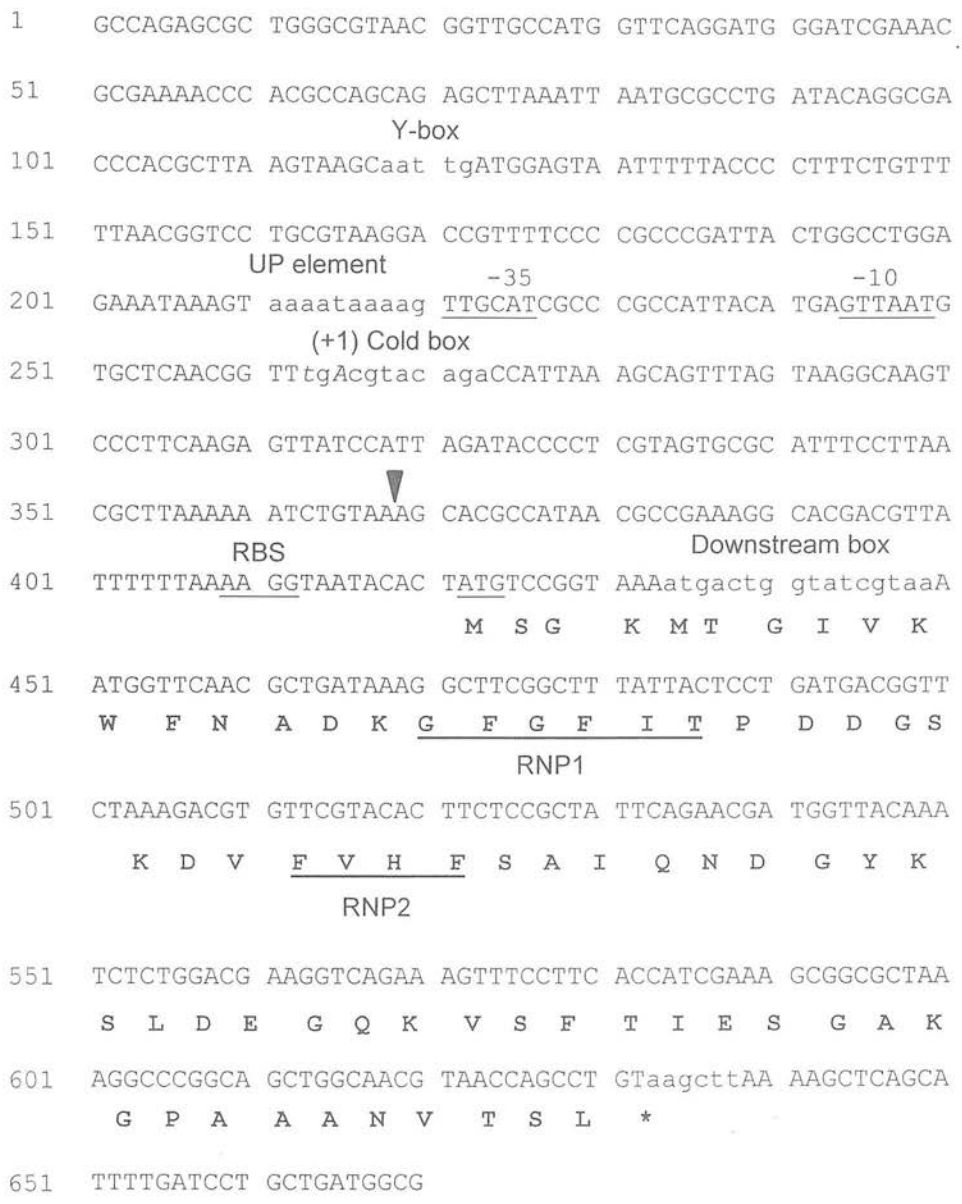


Figure 4.3 DNA sequence of *S. typhimurium cspA*.

The nucleic acid and derived amino acid sequence of *S. typhimurium cspA* (Craig & Chamberlain, unpublished). The putative ribosome binding site, -35 and -10 sites, and translational start sites are underlined, the transcriptional start site is shown in italics. The putative Y-box, UP element, cold box and downstream box regions are shown in lower case. The grey triangle represents the location of the *lacZ* fusion in pNJH6. The *Hind*III restriction enzyme site located at the translation stop codon is also shown in lower case. In the amino acid sequence, the RNP1 and partial RNP2 sequences have been underlined.

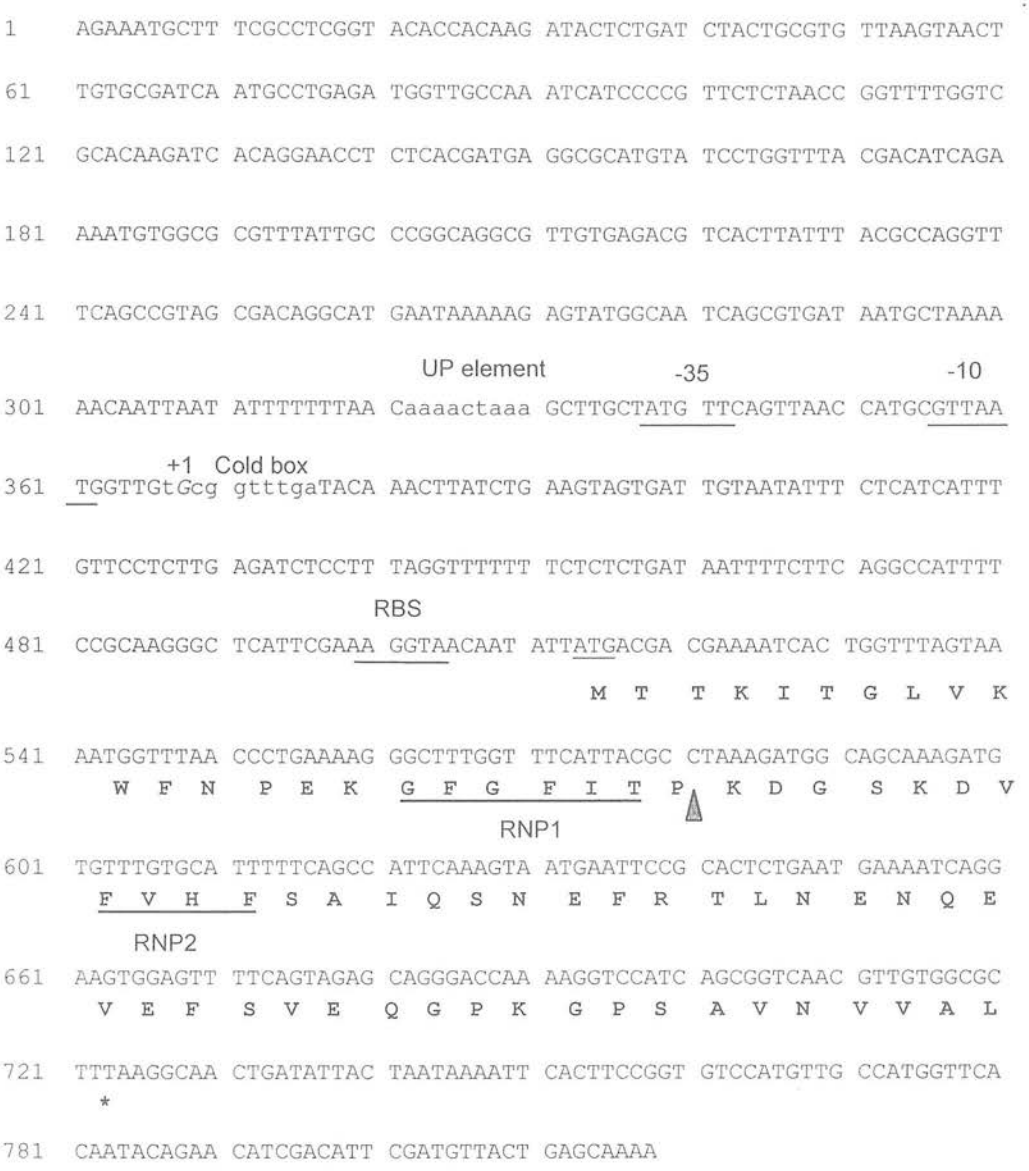


Figure 4.4 Complete DNA and the derived amino acid sequence of *S. typhimurium cspB* (Craig *et al.*, 1998).

The transcriptional start site (mapped by Craig *et al.*, 1998) has been italicised. The putative UP element and cold box are shown in lower case, the –35 and –10 promoter elements, the RBS and the translational start site have been underlined. In the amino acid sequence both RNP motifs have been underlined. The grey triangle represents the location of the Mu*dlux* insertion in strain MPG361.

The *E. coli cspA* fragment contained bases –67 to +143, whilst the *E. coli cspB* fragment contained the *cspB* gene which had the *lacZ* reporter gene fused, in frame, at the 13th codon. In *S. typhimurium cspB*, the sequence similarity for the putative cold box consensus sequence (TGACGTAAGA) is lower than in *S. typhimurium cspA*, such that there are 3 mismatches and 1 deletion. However, the sequence may be sufficient for the cold box to function. In addition, the RNA binding motifs, RNP1 and partial RNP2, are conserved in the amino acid sequences of *S. typhimurium* CspA and CspB. The RNP1 consensus sequence of CspA is KGFGFITR and only the last residue of CspB is different in that proline replaces arginine (Landsman *et al.*, 1992). The partial RNP2 motif of CspB is identical to that of CspA.

The function of CspB is unknown, but due to the high level of DNA and amino acid sequence similarity to CspA, the functions of these 2 proteins may be similar. Bae *et al.* (1997) have shown by 2-dimensional electrophoresis that in an *E. coli cspA* deletion strain, CspB was synthesised at a high level for at least 3 hours following a shift from 37°C to 15°C. This is in contrast to the wild type strain where CspB synthesis peaked and subsequently declined to a low level over this time. This suggests that expression of CspB is prolonged in the deletion strain and so may be able to compensate for the absence of CspA. Similar studies using chromosomal deletion analysis for *B. subtilis*, have suggested that up-regulation of CspB, CspC and CspD may compensate for the lack of one of the other members of the CspA-family, without a reduction in the rate of growth (Graumann *et al.*, 1997).

Reporter gene expression studies in our laboratory, where the bioluminescent *lux* operon from *V. fischeri* has been inserted into the ORF of *S. typhimurium cspB*, have shown that *cspB* is rapidly and highly induced following a reduction in temperature (Craig *et al.*, 1998).

4.1.3 The stress response sigma factor as a global regulator.

Cultures of non-differentiating bacteria, such as *Salmonella*, that have entered stationary phase of growth have a comparable level of resistance to environmental

stresses to those of sporulating bacteria (for review see Kolter *et al.*, 1993). The alternative sigma subunit of RNA polymerase, σ^S, encoded by *rpoS* and expressed during entry into stationary phase, is a global regulator that is necessary for positive regulation of many Pex (post exponential) genes. Additional regulators such as cAMP-CRP and IHF also play key roles on entry to stationary phase. In addition, σ^S is also known to positively regulate a large (and still growing) number of stress response genes, such as those involved in osmotic shock, starvation, oxidative stress, heat shock and DNA damage (for reviews see Loewen & Hengge-Aronis, 1994, Loewen *et al.*, 1998). More recently, RpoS has been shown to accumulate in exponentially growing cells at low temperature in a DsrA-dependent manner. Indeed, the activity of a *rpoS::lacZ* fusion increased 100 fold, following a shift of an exponentially growing culture from 42°C to 20°C (Sledjeski *et al.*, 1996).

4.1.4 Regulation of σ^S

Regulation of σ^S itself, has implications for its role as a regulator and work on *E. coli* has shown that σ^S is regulated at several levels, in a cascade manner by several modulators, as depicted schematically in figure 4.5. This model (adapted from Loewen *et al.*, 1998) is based on assumptions about the most likely explanations for phenotypes and experimental data; future studies are likely to confirm or change some of the information shown. This figure illustrates the complexity of regulation of σ^S.

Levels of σ^S protein reach a maximum during entry into stationary phase, approximately 30 – 50 % that of the level of σ^{70}, which is not growth phase dependent. It is important to bear in mind that when σ^S is induced, other sigma factors, such as σ^{70} are still functional. It appears that the majority of *rpoS* transcriptional expression is σ^{70}-dependent, although CRP may also play a role (Lange *et al.*, 1995).

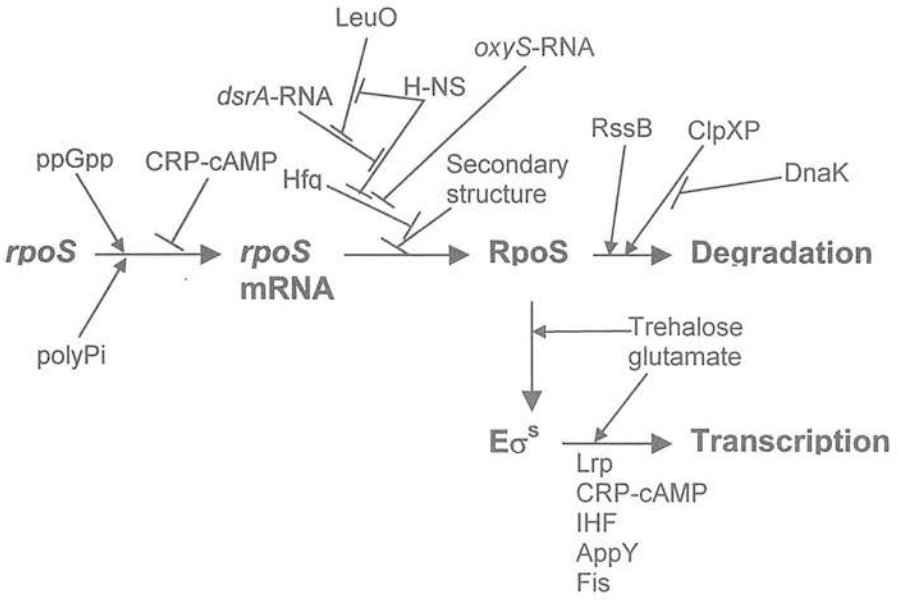


Figure 4.5 Schematic diagram depicting modulators of σ^s activity.

Lines leading from an effector with arrowheads indicate an activating role, lines leading from the effector with a bar at the end indicate an inhibitory role. The effectors listed under the arrow leading from $E\sigma^s$ modulate $E\sigma^s$ transcription of certain genes (Model adapted from Loewen *et al.*, 1998).

Conditions of high osmolarity, low temperature and transition into stationary phase have all been shown to stimulate *rpoS* mRNA translation. Hence the importance of this sigma factor in many stress responses. The secondary structure of *rpoS* mRNA may also play a major role, as the 5' region contains a significant self-complementary sequence that has been predicted to form an extensive branched stem-loop structure. The RBS and initiation codon are located within the predicted secondary structure, thereby making them inaccessible for ribosome binding (Lange & Hengge-Aronis, 1994). Destabilisation of the *rpoS* secondary structure would therefore allow translation. A possible candidate for this is the RNA-binding protein, Hfq, which was initially characterised as an RNA phage host factor. Mutational analysis has shown that the level of Hfq positively correlated with an increase in σ^s accumulation in both exponentially growing and stationary phase cultures (Muffler *et al.*, 1997).

The H-NS protein is a major constituent of the *E. coli* nucleoid and is involved in compaction of the chromosome. It is also one of the proteins induced during cold shock in exponential phase cells and transcription of *hns* appears to be enhanced by CspA binding to a CCAAT sequence in its promoter (Brandi *et al.*, 1994). As a regulator of σ^S, H-NS has been found to bind directly to *rpoS* mRNA (Yamashino *et al.*, 1995) and thereby results in an inhibition of translation, acting in an opposing manner to Hfq. Furthermore, this phenomenon appears to occur independently of growth rate or amino acid availability in minimal medium.

Studies of σ^S in exponentially growing cultures of *E. coli* following a shift from 42°C to 20°C have shown that σ^S accumulation is dependent on a small RNA, *dsrA*. The studies were carried out using an Rpos-LacZ translational fusion in hosts that were either deficient in, or wild type for *dsrA* (Sledjeski *et al.*, 1996). It has been proposed that *dsrA* RNA interacts with H-NS to interfere or antagonise the role that H-NS plays in repression of *rpoS*. It has also been proposed that H-NS plays a negative role in the accumulation of the LysR-like protein, LeuO. Mutation of *leuO* led to a reduction of σ^S at sub-optimal temperature (Klauck *et al.*, 1997). In addition, *oxyS* RNA, which is induced as a major component of the oxidative stress response, has also been shown to reduce σ^S synthesis, possibly by interacting with Hfq (Altuvia *et al.*, 1997).

The protein stability of σ^S provides a further means of regulation and mutation analysis has shown that the ClpXP protease plays a major role in degradation of σ^S protein in exponentially growing cells (Schweder *et al.*, 1996). In addition, the response regulator, RssB, has been shown to have a destabilising effect on σ^S, in response to changes in nutrient availability, either by increasing the rate of ClpXP activity or by enhancing σ^S sensitivity (Loewen *et al.*, 1998). Further studies have shown that DnaK may enhance the stability of σ^S by inhibiting the protease activity of ClpXP (Muffler *et al.*, 1997).

Figure 4.5 also indicates that the regulators Lrp, CRP-cAMP, IHF, AppY, and Fis play roles in modulating the activity of σ^s on some σ^s-dependent target genes. Some of the examples are described below. Lrp, a site specific DNA binding protein termed leucine-responsive regulatory protein, controls a regulon of at least 40 genes in *E. coli*, including σ^s-dependent *aidB* and *osmY* (Landini *et al.*, 1996, Hengge-Aronis 1993). CRP-cAMP has been shown to modulate several σ^s-dependent genes, including *csiA-F* and *glgS* (Marschall *et al.*, 1998, Hengge-Aronis & Fischer, 1992). AppY, part of the acid phosphatase regulon, plays a regulatory role in the expression of σ^s-dependent genes including *appA* and *hyaABCDEF* (Atlung *et al.*, 1997). Fis, a small DNA binding protein whose synthesis increases very rapidly following nutrient enrichment, modulates the activity of negatively regulated σ^s-dependent genes including *glnQ* and *mglA* (Xu & Johnson, 1995).

4.1.5 *E. coli* Fis regulator.

Fis (factor for inversion stimulation) is a small DNA binding protein involved in several biological processes. It is highly conserved in enteric bacteria. Studies of *E. coli* have shown that Fis stimulates phage λ DNA excision and integration, and influences transposition frequencies of transposon Tn*5* and insertion sequence IS*50*. In addition, Fis stimulates transcription of rRNA and tRNA genes (Xu & Johnson, 1995). The carboxy-terminal of the protein includes a helix-turn-helix motif required for DNA binding and bending associated with the stimulation of Hin-mediated DNA inversion, λ DNA excision from the chromosome, and repression of the *fis* promoter. The amino-terminus is uniquely required for Hin-mediated inversion (Beach *et al.*, 1998).

Fis has been shown to negatively regulate expression of various genes in a growth phase-dependent manner. The differential expression of a series of transposon-generated LacZ protein and operon fusions was examined in the presence or absence of Fis (Xu & Johnson, 1995). Two of the strains exhibited a 3 to 4-fold increase in expression in the absence of Fis, in lag and early exponential phase. It is at this point

that Fis expression is at its highest (Ball *et al.*, 1992). The transposon insertions mapped to the *frg* locus in both cases. Several other strains showed an increase in β-galactosidase activity of between 3 and 10-fold, in mid-exponential phase in the absence of Fis. These insertions were found to map to *frg*, *glnQ*, *mglA* and *sdhA*. A similar effect was observed with several other strains in late exponential phase and in stationary phase. These insertions were mapped to the *frg*, *xylF*, and *aldB* and some of these insertions were also found to be RpoS-dependent.

Fis was shown, by measuring β-galactosidase activity of operon fusions, to repress expression of these genes at the RNA level (Xu & Johnson, 1995). Interestingly, some of the genes (of this transposon-generated series) that Fis was shown to repress, encode products required for growth under nutrient-poor conditions, including those involved in transport of xylose, glutamine and methylgalactosides, and the flavoprotein subunit of succinate dehydrogenase (which has also been shown to be cold shock-inducible in *E. coli* (Jones *et al.*, 1987)).

Immediately preceding the *E. coli fis* sequence, in the same operon, is ORF1, which encodes a 321 amino acid protein of unknown function. This ORF and *fis* are co-expressed. The amino acid sequence of *E. coli* Fis is identical to the *S. typhimurium* counterpart. The *S. typhimurium* chromosome also contains ORF1 upstream of *fis*, and it shares 95% identity to *E. coli* ORF1, in putative amino acid sequence. The *fis* promoter region of *S. typhimurium* and *E. coli* has been shown to precede ORF1 (Beach *et al.*, 1998, Osuna *et al.*, 1995).

Fis negatively regulates *fis* mRNA levels by 2.5 fold in *S. typhimurium*, by binding to sites I and II, thereby excluding RNA polymerase binding. In *E. coli*, Fis also binds, less strongly, to 4 additional sites which are not conserved in *S. typhimurium* (Osuna *et al.*, 1995). In addition, *E. coli fis* transcription is enhanced approximately 3.8-fold through IHF binding to a site upstream of Fis sites I and II. The IHF site is reasonably well conserved in *S. typhimurium* and therefore would most likely have the same regulatory function (Beach & Osuna, 1998).

During the lag period, following a nutrient upshift, Fis is highly and rapidly induced, reaching peak levels of 40,000 dimers per cell. Once exponential growth begins, Fis protein and mRNA levels decrease, reaching low levels in exponential phase. *E. coli* cells that have been in stationary phase for at least 10 hours contain less than 100 copies of Fis protein per cell (Ball *et al.*, 1992). An identical region in both *S. typhimurium* and *E. coli*, between –49 and +94 (relative to the transcriptional start site), appears to be responsible for the observed high level of *fis* expression following a nutrient upshift, as shown by Fis protein and mRNA levels (Osuna *et al.*, 1995). In addition, *fis* expression has been shown to be stringently controlled, by using a test for stringently activated promoters. For this test, the cells were grown in minimal media, supplemented with 18 amino acids but lacking in isoleucine and valine, which induced a stringent response. Chloramphenicol was subsequently added to the bacteria which led to an abrupt decrease in the level of ppGpp due to the accumulation of aminoacylated tRNAs. At this point, the activity of stringently controlled promoters is restored. (Ninnemann *et al.*, 1992).

4.1.6 Bioluminescence *lux* genes.

Light reporter studies make use of bioluminescence *lux* genes derived from various marine bacteria including *Vibrio, Photobacterium* and *Xenorhabdus*. Although differences exist between *lux* genes of the different genera, the functions are essentially the same. The light producing reaction involves oxidation of reduced riboflavin phosphate ($FMNH_2$) and a long chain fatty aldehyde with the emission of blue/green light. The reaction is catalysed by luciferase shown in figure 4.6.

$$FMNH_2 + O_2 + R.CHO \xrightarrow[luciferase]{} FMN + H_2O + R.COOH + h\nu_{(490nm)}$$

Figure 4.6 The chemistry of the production of bioluminescence.

Luciferase is the gene product of *luxA* and *luxB* which form the α and β subunits of the protein, respectively. The synthesis of the aldehyde for the bioluminescence reaction is catalysed by a multi-enzyme fatty acid reductase made up of 3

components; a reductase, a transferase and a synthetase encoded by *luxC, luxD* and *luxE*, respectively. The *lux* genes of *Vibrio fischeri* are organised as follows (figure 4.7).

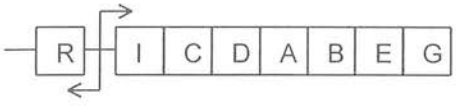


Figure 4.7 Structural organisation of the *lux* genes of *Vibrio fischeri* (not drawn to scale), (Meighen, 1991).

Both *luxR* and *luxI* are involved in regulation of the *lux* operon; they encode a regulator protein and an autoinducer, respectively. The function of *luxG* is unknown, but it may be involved in a function associated with the marine environment (Meighen, 1991).

For reporter purposes, *luxCDABE* genes from *V. fischeri* are fused to a promoter of interest and bioluminescence is measured at 490nm, giving a real time determination of reporter activity. This light reporter system has been used in this study to monitor gene expression of *S. typhimurium cspB* following a temperature reduction.

4.1.7 Aims of the chapter.

The main aim of this section was to characterise thermoregulation of *S. typhimurium* cold shock genes, *cspA* and *cspB*, at low temperature and to determine whether factors such as growth phase or global regulators played a role in their regulation. Expression of *S. typhimurium cspA* was monitored by measuring β-galactosidase activity from a plasmid-borne transcriptional *cspA-lacZ* fusion. The plasmid carrying the *cspA-lacZ* fusion was harboured in *E. coli* cells and β-galactosidase activity was measured following a shift from 37°C to 15°C.

The expression of *cspB* was monitored by measuring bioluminescence from a light-based reporter system that was translationally fused to *cspB*, on the chromosome, in

strain MPG361 (*cspB::*Mu*dlux*). Bioluminescence from this strain was measured at 30°C, 10°C and 4°C, either when the cells were exponentially growing or in stationary phase. The low temperatures that were used for investigation of *cspB* expression, 10°C and 4°C, represent temperatures both above and below the minimum temperature permissible for growth, respectively. In particular, 4°C was chosen to mimic food storage conditions since refrigeration of food is commonly carried out at 4°C. At this temperature, growth of many pathogenic bacteria, including *Salmonella* is not possible (Mossel *et al.*, 1981). Very few studies have been carried out on gene expression under these conditions.

There are an increasing number of reports of the regulatory role of the alternative sigma factor, σ^s, involved in stress responses. Given these reports, it was appropriate to determine whether σ^s played a regulatory role in *S. typhimurium cspB* expression following a downshift in temperature. As yet, σ^s has not been implicated in the regulation of the cold shock response at low temperatures. A *rpoS::bla* disruption (SF1005) was transduced into the chromosome of MPG361 (*cspB::*Mu*dlux*) using P22 transduction, forming strain MPG480. This strain was used for bioluminescent reporter studies of *cspB* expression at various temperatures and under different growth phases.

The DNA binding protein, Fis, has been shown to be involved in regulation of various systems, including enhancement of transcription of rRNA and tRNA genes (Nilsson *et al.*, 1990). In addition, Fis has been shown to repress expression of several different genetic loci, in a growth-phase dependent manner (Xu & Johnson, 1995). In particular, the expression of *sdhA*, measured from a transposon-generated LacZ protein fusion, increased 3-fold in the absence of Fis, when the cells were in mid-exponential phase. This is interesting since SdhA has also been shown to be cold shock inducible in *E. coli* cells that were in exponential phase (Jones *et al.*, 1987). Thus it was important to determine whether Fis played a role in the regulation of expression of *S. typhimurium cspB*. This was carried out by transducing a *fisΔcml* mutation from strain RJ1829 (Osuna *et al.*, 1995), onto the chromosome of MPG361

(*cspB::*Mu*dlux*), using P22 transduction, forming strain MPG481. This strain was used for bioluminescent reporter studies of *cspB* expression at various temperatures and under different growth phases.

4.2 RESULTS

4.2.1 Gene expression of *S. typhimurium cspA* using a plasmid-borne transcriptional *cspA-lacZ* fusion.

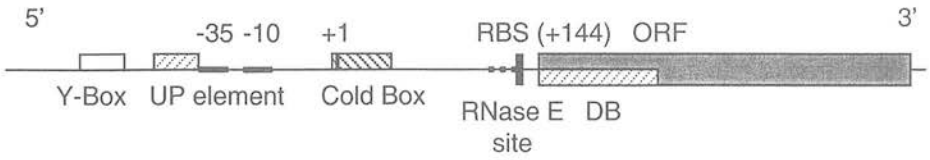
Gene expression from the *S. typhimurium cspA* locus was examined using a plasmid-based transcriptional *cspA-lacZ* fusion, in *E. coli* host cells, at low temperature. Use of *cspA-lacZ* fusions in *E. coli* have given conflicting results in terms of equating the levels of β-galactosidase activity with regulation of CspA production (Goldenberg *et al.*, 1996; Lee *et al.*, 1994), although in each case, gene expression from the fusion increased when the culture was incubated at low temperature. One group found that gene expression from both transcriptional and translation *cspA-lacZ* fusions increased approximately 2.5-fold following incubation at 15°C for 4 hours (Goldenberg *et al.*, 1996), whilst a second study reported that β-galactosidase activity increased 18-fold from a translational fusion following a similar incubation at 15°C (Lee *et al.*, 1994). In the first case, the plasmid that contained the *cspA-lacZ* fusion was harboured in a *pcnB* strain, that is known to maintain a low number of pBR322 derived plasmids. No such mutation was used in the second report.

In order to explore the situation for the *cspA* gene of *S. typhimurium*, the *lacZ* gene carrying its own RBS was amplified by PCR from EMG2 (bases 1232 – 4340) using primers LAC5 and LACZR (table 2.3, chapter 2 of this thesis). The primers incorporated artificial *Bgl*II and *Hind*III restriction sites, respectively, to facilitate sub-cloning into the corresponding restriction sites in pNJH5. This located the *lacZ* gene at base 380 of *cspA*, which is +105 bp from the transcriptional start site of *cspA* (illustrated in figure 4.4) The RBS and translational start site of *cspA* were therefore absent from the *cspA-lacZ* fusion. The resulting plasmid, pNJH6 (shown in figure 3.5 A) was transformed into a variety of *E. coli* hosts and the promoter activity was observed by measuring β-galactosidase activity. One such host, MM38, carries a *pcnB* mutation (*pcnB::kan*) which reduces the copy number of ColE1-based plasmids, such as pBR322 to approximately 1 – 3 copies per cell (Lopilato *et al.*,

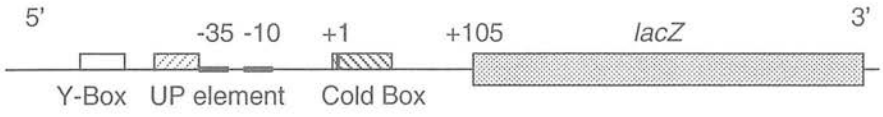
1986). This low copy number more realistically reflects the promoter activity of *cspA in vivo*. In addition, this host strain is *lacZ* negative, so that the only source of β-galactosidase activity was from the *cspA-lacZ* fusion. β-galactosidase activity was also monitored from DH5α, EMG2 (a wild type *E. coli*) and DH5α harbouring pBR322. A comparison of the genetic features in the *cspA-lacZ* constructs with the native *S. typhimurium cspA* locus is shown in figure 4.8.

Measurement of β-galactosidase from the *cspA-lacZ* fusion was essentially as described by Miller (1972). In short, cultures were grown in LB media, with shaking, to mid exponential phase before being incubated at 15°C for 5 hours. Triplicate Samples of 0.5 ml were collected at hourly intervals and the enzyme activity was measured as described in chapter 2 of this thesis. For each time point, samples were also collected and processed without enzyme substrate to act as blanks for spectrometer measurements. The results are shown in figure 4.9.

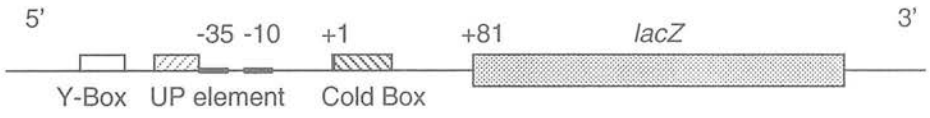
Following incubation at 15°C, β-galactosidase activity from the *cspA-lacZ* fusion (pNJH6) in host MM38, was shown to increase 8-fold over a 5 hour period (figure 4.9). The effect of reducing the plasmid copy number was demonstrated by monitoring β-galactosidase levels from pNJH6 in the host DH5α (this host strain is also *lacZ* negative), which resulted in a 20-fold increase in β-galactosidase activity, relative to pNJH6 in host stain MM38, following the temperature downshift. As a negative control, β-galactosidase levels were also measured from the pBR322 plasmid vector (in strain DH5α). In addition, β-galactosidase activity was monitored from a wild type *E. coli* strain, EMG2, which was both induced using a lactose analogue, IPTG, or uninduced, to determine the effect of incubation at 15°C on a non-cold inducible (chromosomally-based) gene. The level of β-galactosidase activity from the culture of induced EMG2 increased 1.3-fold after incubation for 3 hours at 15°C and decreased to its lowest level after 5 hours at 15°C. The level of β-galactosidase activity from the uninduced EMG2 culture increased 5-fold after 4 hours at 15°C, although activity subsequently decreased after a further hour at 15°C.



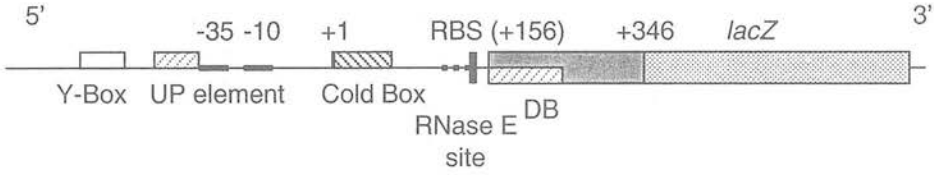
A. *S. typhimurium cspA* and the putative regulatory elements (not drawn to scale).



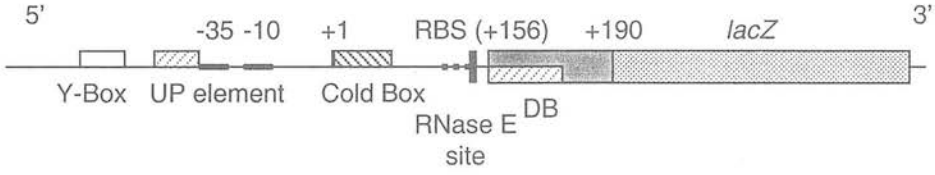
B. The *S. typhimurium cspA-lacZ* transcriptional fusion present in pNJH6 (this thesis).



C. The *E. coli cspA-lacZ* transcriptional fusion, chromosomally integrated at the λ phage attatchement site (Goldenberg *et al.*, 1996).



D. The *E. coli cspA-lacZ* translational fusion, chromosomally integrated at the λ phage attatchement site (Goldenberg *et al.*, 1996).



E. The *E. coli cspA-lacZ* translational fusion, plasmid based in a *pcnB* host (Lee *et al.*, 1994).

Figure 4.8 Genetic organisation of the *cspA* locus.

Schematic diagrams of the *S. typhimurium cspA* locus, together with its associated putative regulatory elements (A), and various *cspA-lacZ* fusions (B – E) (not drawn to scale). The putative UP element, cold box and the downstream regulatory box (DB) are indicated by hashed boxes. The core Y-box sequence is indicated by an open box, the putative RNase E site is indicated by a dotted line. The putative –35, -10, +1 and RBS are indicated by heavy lines. *lacZ* is indicated by a shaded box and the *cspA* ORF is indicated as a solid box.

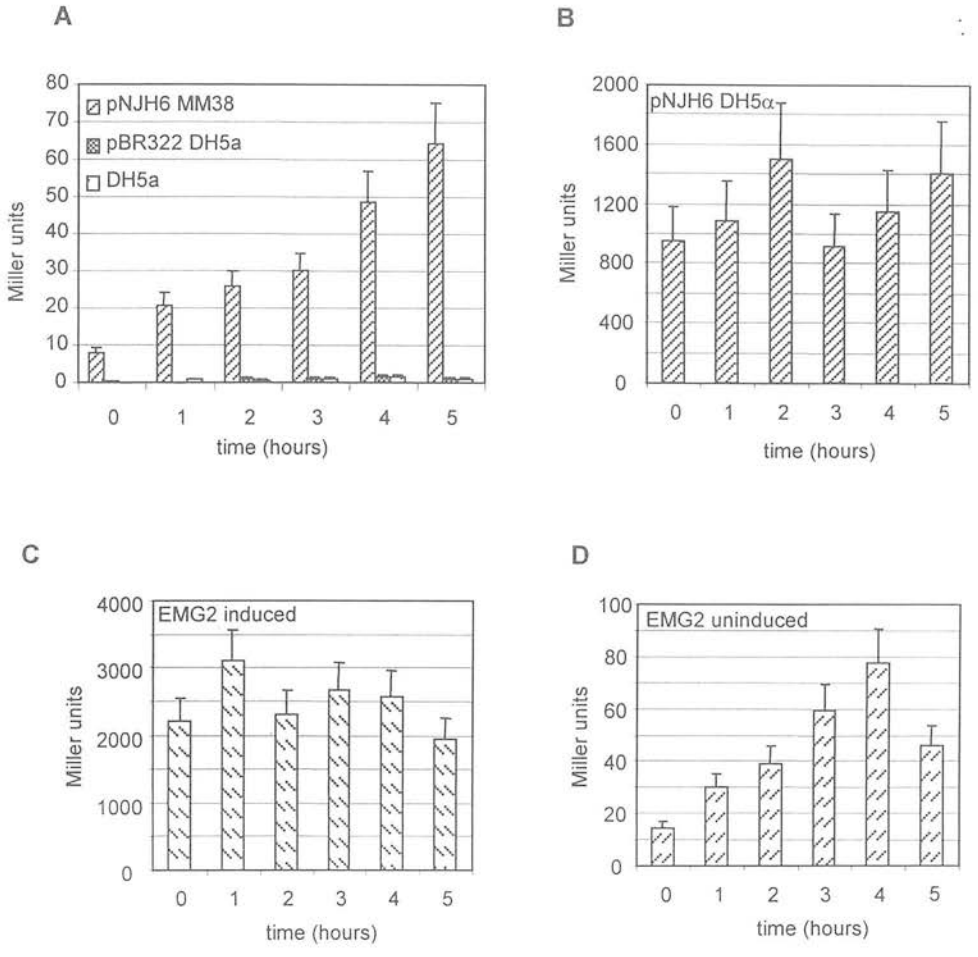


Figure 4.9 β-galactosidase measurements (Miller units) from *S. typhimurium cspA-lacZ*.

A, the plasmid pNJH6 was harboured in MM38, plasmid pBR322 was harboured DH5α and strain DH5α was included as a negative control.
B, the plamid pNJH6 was harboured in strain DH5α.
C, strain EMG2 was induced with 1 mM ITPG.
D, strain EMG2 was uninduced.
The cultures were grown at 37°C to mid-logarithmic phase and incubated at 15°C for a total of 5 hours. Samples were taken, in triplicate, at hourly intervals, including at 0 hours which represents 37°C.

Taken together, these results show that activity from the *S. typhimurium cspA* promoter increased over a 5 hour period when exponentially growing cells were incubated at 15°C. However, activity of a non-cold inducible gene also increased over this period, although the increase was less than observed for *cspA*.

4.2.2 Thermo-regulation of *S. typhimurium cspB*, in exponential phase.

Previously in our laboratory, construction of a pool of Mu*dlux* fusions led to insertion of the bioluminescent *luxCDABE* genes from *V. fischeri*, into the 23rd codon of *S. typhimurium cspB*. This strain, MPG361 (*cspB::*Mu*dlux*) was used to monitor expression of *cspB* by measuring light production in a luminometer. Expression of *cspB* was monitored when the culture was in exponential phase of growth at 30°C, and following shifts to low temperature, either above the minimum growth temperature at 10°C, or below the minimum growth temperature at 4°C.

The method used to monitor bioluminescence is essentially as described in Chapter 2 of this thesis. Bioluminescence was measured in triplicate from 0.2 ml samples of MPG361 (*cspB::*Mu*dlux*), MPG480 (*cspB::*Mu*dlux, rpoS::bla*), or MPG481 (*cspB::*Mu*dlux, fis::cml*) at 30°C, 10°C or 4°C for 24, 72 and 96 hours, respectively. The cultures were either in late exponential phase or in stationary phase of growth. The luminometer (Luminoskan RS) and the microtitre plates were equilibrated to the specified temperature for at least 1 hour before light production measurements were taken. As a negative control, bioluminescence from 3 wells with 200 µl of LB was measured. No bioluminescence was detected from these negative control wells during any of the experiments.

At the start of the light production measurements, the number of c.f.u. (colony forming units) was estimated by spreading dilutions of the culture onto LB agar containing the appropriate antibiotic. The bioluminescent measurements are given as arbitrary RLU (relative light units) per bacterial cell.

It should be noted that although the *lux* reporter system is valuable for determining gene expression, it is known that the *lux* gene product can be stable over a period of hours. Thus, it may give a false impression of delayed decay, in comparison to the half life of the native gene product. In this set of experiments, the half life of the *cspB*-luciferase fusion product was not measured in comparison to the native CspB protein. However, the primary aim of this chapter was to examine the low temperature regulation of *cspB*, therefore, the level of induction of bioluminescence from the *cspB::*Mu*dlux* fusion was the primary concern.

4.2.2.i Expression of *cspB* at 30°C and 10°C.

Maintenance of a culture of MPG361, that was in late exponential phase of growth, at 30°C, resulted in no detectable bioluminescence (figure 4.10). The starting number of colonies was approximately 2 x 10^8 c.f.u ml^{-1}. This is in line with the results of Craig and colleagues (1998), that *S. typhimurium cspB* was not expressed above a threshold temperature of 22°C. However, shifting the culture from 30°C to 10°C resulted in induction of *cspB::*Mu*dlux* within 90 minutes (figure 4.11) and the level of bioluminescence increased at a constant rate for approximately 15 hours. These conditions resulted in the maximum level of observed bioluminescence from strain MPG361, during all the conditions tested in this study. Thus, *cspB* was most highly induced following a temperature downshift from 30°C to 10°C, when the cells were in late exponential phase.

Figure 4.10

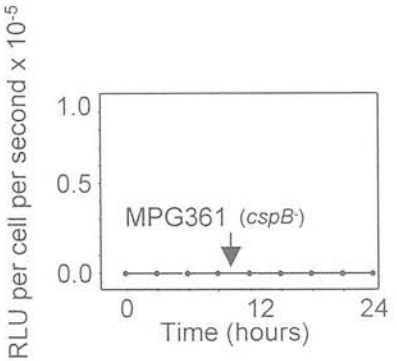


Figure 4.11

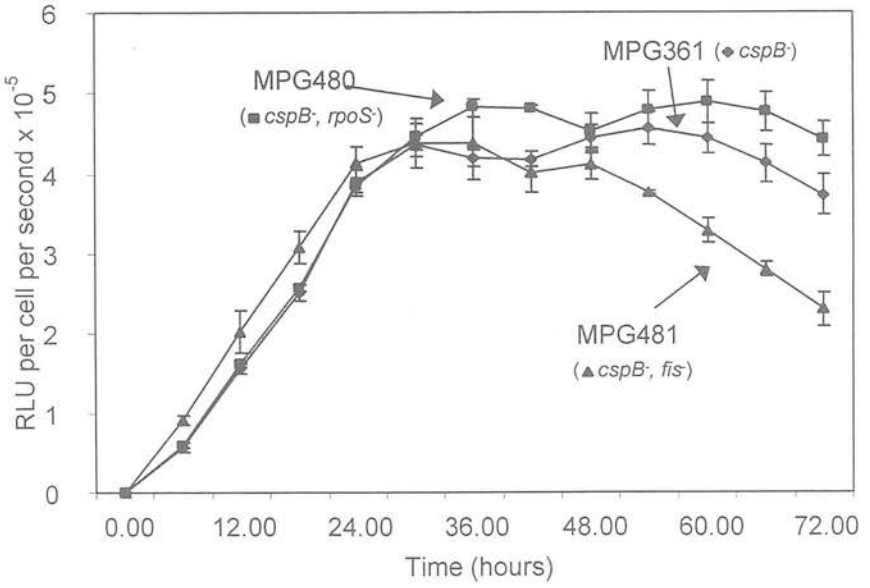


Figure 4.10 Expression of *S. typhimurium cspB* at 30°C, in exponential phase.
Figure 4.11 Expression of *S. typhimurium cspB* following a shift from 30°C to 10°C, in exponential phase.

Strains MPG361, MPG480 and MPG491 were grown to late exponential phase, at 30°C and either maintained at 30°C (figure 4.10) or shifted to 10°C (figure 4.11). Samples were pipetted into a white, 96-well micro-titre plate and placed into a luminometer that had been pre-equilibrated to 30°C or 10°C. Bioluminescence was measured immediately and then every 15 minutes, for a total of 24 hours at 30°C or 72 hours at 10°C.

4.2.2.ii Expression of *cspB* below the minimum temperature permissible for growth, at 4°C.

Expression of *cspB* from strain MPG361 at 4°C was also examined. Investigation of gene expression at this temperature is especially important since although it is known that proliferation of *S. typhimurium* does not occur (Mossel *et al.*, 1981), there are very few reports on gene expression under such conditions. Previous studies have shown that *E. coli cspB* was expressed at 6°C, (which is below the minimum temperature permissible for growth of *E. coli*), although the levels of *cspB* mRNA and CspB protein were both found to be extremely low (Etchegaray *et al.*, 1996). At 6°C, the level of CspB protein was found to be only 10% that of the level produced following a shift from 37°C to 15°C, (the optimum temperature for *E. coli* CspB production) (Etchegaray *et al.*, 1996)).

Shifting an exponentially growing culture of MPG361 from 30°C to 4°C resulted in a delay of several hours before bioluminescence was detected (figure 4.12). The starting number of colonies was approximately 2 x 10^8 c.f.u ml^{-1} . Light production increased with time, but at a much lower rate and to a 10-fold lower level than observed for a similar culture that had been shifted to 10°C. Thus, these experiments show that expression of *cspB* occurs to some degree when an exponential culture is incubated at 4°C for several hours, although after a delay of several hours.

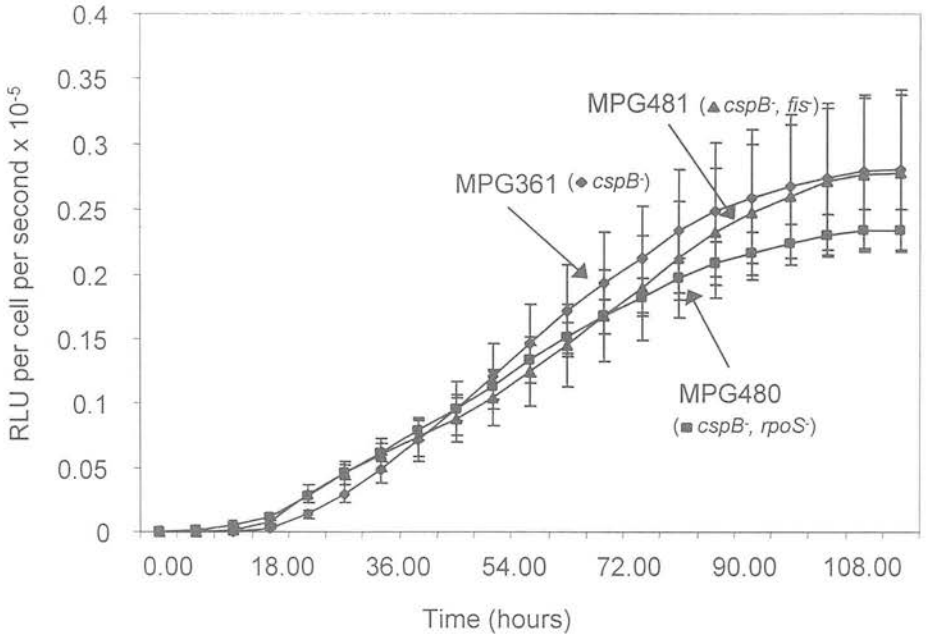


Figure 4.12 Expression of *S. typhimurium cspB* following a shift from 30°C to 4°C, in exponential phase.

Strains MPG361, MPG480 and MPG491 were grown to late exponential phase, at 30°C and shifted to 4°C. Samples were pipetted into a white, 96-well micro-titre plate and placed into a luminometer that had been pre-equilibrated to 4°C. Bioluminescence was measured immediately and then every 15 minutes, for a total of 96 hours.

4.2.3 Thermo-regulation of *S. typhimurium cspB*, in stationary phase.

Thermo-regulation of MPG361 was also investigated in cells that had entered stationary phase. The starting number of colonies was 8 x 10^9 c.f.u ml^{-1}. The change in growth phase from exponential growth to stationary phase is known to affect the expression of many genes (Lange & Hengge-Aronis, 1991), therefore it was important to determine whether growth phase also affects thermo-regulation of *cspB*. Little has been reported about the cold shock response when cells are in stationary phase or how gene expression of cold shock genes is affected under these conditions.

4.2.3.i Expression of *cspB* at 30°C and 10°C.

Expression of *cspB* from strain MPG361 was monitored at 30°C, when the culture was in stationary phase. The starting number of colonies was 8 x 10^9 c.f.u ml^{-1} . Similar to the findings for cultures that were in exponential phase, no bioluminescence was detected at this temperature (figure 4.13). However, shifting the culture from 30°C to 10°C resulted in induction of bioluminescence after a delay of approximately 3 hours (figure 4.14). Light production continued to rise for several hours. The level of *cspB* expression was approximately 10-fold lower when the cells were in stationary phase in comparison to exponentially growing cultures of MPG361.

Figure 4.13

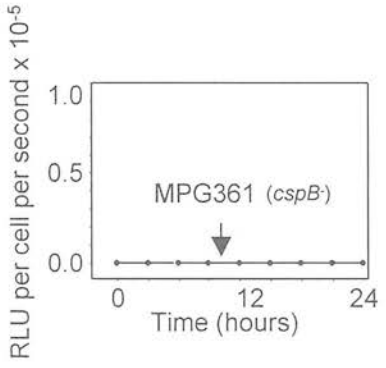


Figure 4.14

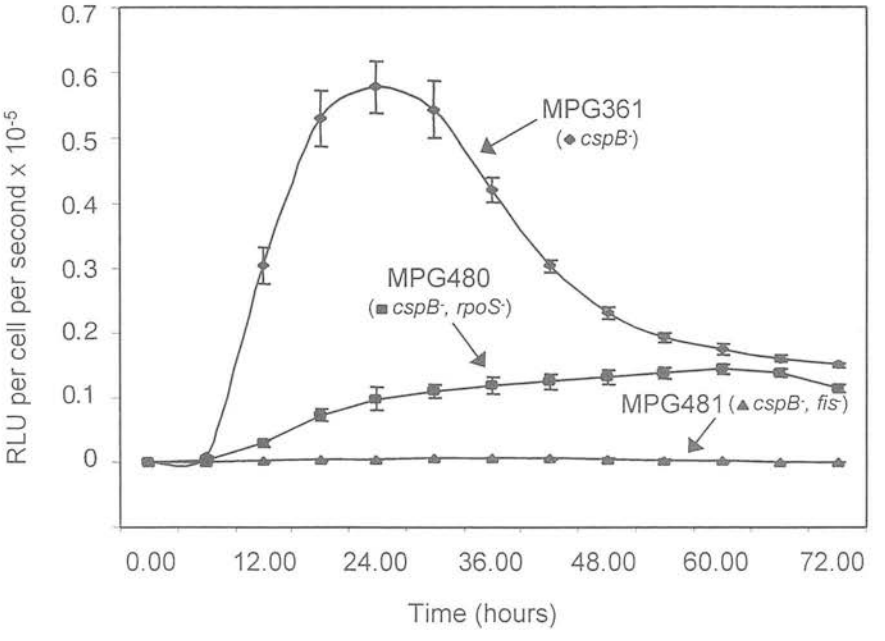


Figure 4.13 Expression of *S. typhimurium cspB* at 30°C, in stationary phase.
Figure 4.14 Expression of *S. typhimurium cspB* following a shift from 30°C to 10°C, in stationary phase.

Strains MPG361, MPG480 and MPG491 were grown to stationary phase, at 30°C and either maintained at 30°C (figure 4.13) or and shifted to 10°C (figure 4.14). Samples were pipetted into a white, 96-well micro-titre plate and placed into a luminometer that had been pre-equilibrated to 10°C. Bioluminescence was measured immediately and then every 15 minutes, for a total of 24 hours at 30°C or 72 hours at 10°C.

4.2.3.ii Expression of *cspB* below the minimum temperature permissible for growth, at 4°C.

Expression of *cspB* was investigated below the minimum growth temperature, when the cells were in stationary phase. A delay of approximately 18 hours occurred before bioluminescence was detected (figure 4.15). Light production then increased, at a slow rate, for the remainder of the incubation. The level of light production was also very low, approximately 2 orders of magnitude lower than observed in a similar culture at 10°C. Thus, expression of *cspB* appears to be minimal below the minimum growth temperature, during stationary phase.

4.2.4 The role of global regulators on the expression of *S. typhimurium cspB*

The role of the global regulators, σ^s and Fis, were assessed in the cold shock response of *S. typhimurium* by monitoring the expression of *cspB* in the absence of these factors. Both regulators have been shown to affect gene expression throughout growth phase, thus their effects were assessed in both exponentially growing and stationary phase cells.

4.2.4.i Regulation of *S. typhimurium cspB* by RpoS.

The regulatory effects of *rpoS*, which encodes an alternative sigma factor, were examined in strain MPG480 (*cspB::*Mu*dlux rpoS::bla*). This strain contains an insertional inactivation of the *rpoS* gene in addition to the *cspB::*Mu*dlux* fusion. Thus, light production from this strain reflects the effect, if any, of the alternative sigma factor σ^s. It has been reported that in addition to the regulation of at least 30 post-exponential genes (Loewen & Hengge-Aronis, 1994), σ^s is also involved in regulation of several stress response genes (Muffler *et al.*, 1997; Hengge-Aronis, 1996).

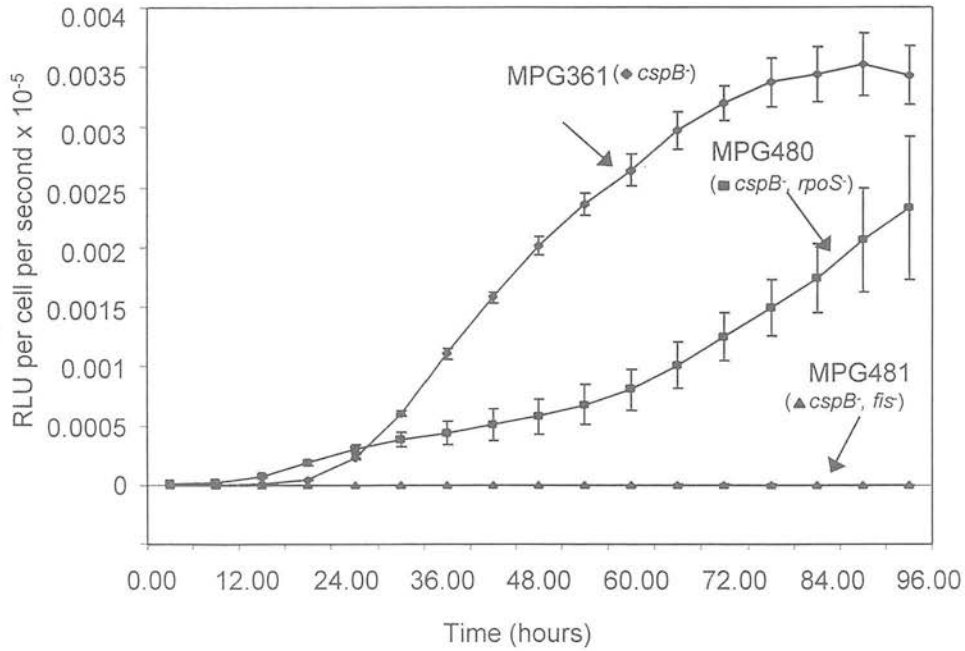


Figure 4.15 Expression of *S. typhimurium cspB* following a shift from 30°C to 4°C, in stationary phase.

Strains MPG361, MPG480 and MPG491 were grown to stationary phase, at 30°C and shifted to 4°C. Samples were pipetted into a white, 96-well micro-titre plate and placed into a luminometer that had been pre-equilibrated to 4°C. Bioluminescence was measured immediately and then every 15 minutes, for a total of 96 hours.

Moreover, RpoS has also been shown to be induced following a shift from 37°C to 20°C, although this low temperature induction was found to be dependent on a small RNA encoded by *dsrA* (Sledjeski *et al.*, 1996). Thus, the role of σ^s was determined in the low temperature induction of *S. typhimurium cspB*.

The effect of *rpoS* on *cspB* expression was determined in exponentially growing cells. However, since the cultures were in late logarithmic phase of growth, *rpoS* is likely to have been induced. The starting number of colonies was 4 x 10^8 c.f.u ml^{-1} . The σ^s sigma factor is induced following entry into stationary phase in addition to induction following several environmental stresses (Loewen & Hengge-Aronis 1994). Cells that have not been exposed to environmental stress and that are in exponential growth phase exhibit repressed expression of *rpoS* (Yamashino *et al.*, 1995). Thus, the status of *rpoS* cannot be confirmed as being repressed during these experiments.

Expression of *cspB* in late exponential phase cultures was monitored following a temperature decrease from 30°C to 10°C, in the absence of *rpoS* (strain MPG480) (figure 4.11). Bioluminescence from strain MPG480 was in line with that observed from MPG361. Thus, *rpoS* did not appear to play a role in regulation of *cspB* at 10°C. Similarly, bioluminescence detected from strain MPG480 following a shift from 30°C to 4°C approximated that observed from MPG361 (figure 4.12).

The role of *rpoS* in the thermo-regulation of *cspB* was also investigated when the cells were in stationary phase. The starting number of colonies was 10 x 10^8 c.f.u ml^{-1}. Shifting a culture of MPG480 from 30°C to 10°C resulted in a 5-fold lower level of bioluminescence in comparison to that observed from MPG361, after 24 hours incubation at 10°C (figure 4.14). Similarly, shifting a culture of MPG480 from 30°C to 4°C also resulted in a lower level of bioluminescence, as compared to that from MPG361 (figure 4.15). However, since the level of bioluminescence detected at this temperature was very low in comparison to that detected from the culture at 10°C, these results should be viewed cautiously. These results suggest that the

presence of *rpoS* increased the expression of *cspB* following shifts to low temperature, both above and below the minimum temperature permissible for growth, when the cells were in stationary phase.

4.2.4.ii Regulation of *S. typhimurium cspB* by *E. coli* Fis.

In addition to investigating the regulation of *cspB* by the alternative sigma factor, σ^s, at low temperature, regulation by a second global regulator, Fis, was also studied, by the same method as described above. Since a decrease in temperature results in a block in translation initiation, it was thought that Fis may be up-regulated during this time, or may up-regulate the cold shock genes. Fis regulation of *cspB* was investigated using the *cspB::*Mu*dlux* strain which also contained an insertional inactivation of *fis* by the chloramphenicol cassette, strain MPG481. The effect of growth phase was also studied, as described previously. However, since the cell density of the cultures that were in late exponential phase was relatively high (approximately 2 x 10^8 cfu ml^{-1}), the level of Fis would be relatively low. Similarly, the level of Fis would be expected to be less than 1000 copies of protein per cell for the cells that were in stationary phase (Osuna *et al.*, 1995).

Cultures of MPG481 that were in late exponential phase were shifted from 30°C to 10°C and the level of bioluminescence from the *cspB::*Mu*dlux* reporter system measured. Light production from this strain was very similar to that from MPG361 (figure 4.11), which indicated that *cspB* expression was not affected by Fis in late exponential phase. In a similar manner, light production from cultures of MPG481, also in late exponential phase, that were shifted from 30°C to 4°C in line with that produced from MPG361 (figure 4.12).

In contrast, cultures of MPG481 that had been incubated at 30°C for 24 hours prior to a downshift to 10°C, resulted in a very low level of light production. The level of light detected from MPG481 was always lower than that from MPG361 (figure 4.14). Moreover, after incubation for 18 hours at 10°C, light production from MPG481 was

less than 2 orders of magnitude than that observed from MPG361. More striking was the fact that no bioluminescence was detected from MPG481 when the stationary phase culture was shifted from 30°C to 4°C (figure 4.15). Thus, Fis appears to play a role in the regulation of *cspB*, in a growth phase dependent manner.

4.3 Discussion

During the adaptation period of the cold shock response, the majority of protein synthesis is repressed whilst at the same time induction of a subset of cold shock inducible proteins occurs, including induction of some of the CspA homologues. Regulation of cold shock inducible *cspA* homologues has been studied in detail for *E. coli* genes. In particular, mRNA stability of the major cold shock gene appears to play a major role in cold shock regulation, as observed in *E. coli* and *S. typhimurium*. However, differential regulation of *E. coli cspA* and *cspB* has been observed, such that the former was shown to be induced over a broader temperature range than the latter (Etchegaray *et al.,* 1996). In addition, functional differences have also been shown to exist between the major cold shock protein of *B. subtilis* and the other, less highly induced, cold shock proteins, in terms of binding affinities to nucleic acids (Graumann *et al.*, 1997)

An UP element located upstream of the –35 region of a number of bacterial promoters, has previously been reported to stimulate transcription (Ross *et al.*, 1993). A similar UP element in *E. coli cspA*, located between –47 to –38 (AAAATAATTG), has been implicated in enhancing *cspA* promoter activity, following downshift to 10°C. Induction of *cspA* has been shown to be abolished when the region upstream of the *cspA* promoter, (from bases –69 to –39, with respect to the transcriptional start site), is deleted (measured from transcriptional *cspA-lacZ* fusion constructs). In contrast, induction of *cspA* was shown to be approximately 20-fold, following a 3 hour incubation at 15°C, when this region was intact (Mitta *et al.*, 1997). It appeared that DNA containing the UP element was responsible for the enhanced promoter activity. *S. typhimurium* also seems to contain an UP element in the *cspA* promoter, which is identical to that of *E. coli cspA*, both in terms of the nucleic acid sequence and position in relation to the promoter region (shown in figure 4.3).

A further factor has been shown to be important in the expression of cold shock-inducible genes. The translational efficiency of *E. coli cspA* appears to be

substantially higher than that of non-cold shock inducible genes (Mitta *et al.*, 1997). The *cspA* translation initiation region (from bases +144 to +198, with respect to the transcriptional start site) was replaced with that of a *lpp-lacZ* fusion construct. Reporter activity from the construct containing the *cspA* fragment was 3-fold higher than that from the *lpp-lacZ* construct. This region of *cspA* included the so-called downstream box (DB). Previously, it has been suggested that the DB interacts with the complementary sequence of 16S rRNA and mediates efficient translation from the initiation codon, independently of the RBS (Sprengart *et al.*, 1996). In *E. coli cspA*, the DB is a region which is comprised of the initial 10 codons of the translational sequence (figure 4.2) (Mitta *et al.*, 1997). Indeed, in one study where part of the DB of *E. coli cspA* was deleted, this resulted in a 50% reduction in production of a CspA-β-galactosidase fusion protein. Again, because this region of *E. coli cspA* is identical to that of *S. typhimurium*, the putative regulation is likely to be the same for both organisms.

In contrast, the cold box, an 11bp motif with a sequence of UGACGUACAGA (from *E. coli cspA*), has been shown to repress *E. coli* CspA and CsdA production after 3 hours at 15°C (Fang *et al.*, 1998). The cold box sequence of *cspA* and *cspB* of *S. typhimurium* and *cspA, cspB* and *csdA* of *E. coli* are shown in figure 4.16. The cold box from *S. typhimurium cspA* is identical to that of *E. coli cspA*, although the *S. typhimurium* cold box is positioned slightly upstream of the *E. coli* cold box, with respect to the transcription start positions. However, the cold box region of *S. typhimurium cspB* contains 3 mismatches relative to the *E. coli cspA* cold box, which suggests that the cold box does not play such an important role in regulation of CspB in *S. typhimurium*.

Studies with *E. coli cspA* have also revealed a putative RNase E binding site that is formed in the mRNA secondary structure, close to the initiation codon. Substitution of the 3 bases in the RNase E site lead to a 150-fold increase in *cspA* mRNA stability at 37°C. However, other factors are evidently involved in degradation of *cspA* RNA at optimal temperatures, since the 3 base substituted *cspA* mRNA was only partially stabilised in a rne^{ts} background (Fang *et al.*, 1997).

UGACGUACAGA	Cold Box consensus
ACGGTTUGACGUACAGACCAUUAA	*S. typhimurium cspA* -7 to +17
TGGTTGUG CGGUUUGAUACAAAC	*S. typhimurium cspB* -7 to +16
ACGGUUUGACGUACAGACCAUUAA	*E. coli cspA* +1 to +24
UCGGUUUGAAGAACAGACGAUAUA	*E. coli cspB* +2 to +25
AAUAGCUGACGUACACAAUCAGCC	*E. coli csdA* +42 to +65

Figure 4.16 Alignment of the *S. typhimurium* and *E. coli* cold box sequences.

The cold box sequences of *S. typhimurium cspA* and *cspB*, and *E. coli cspA, cspB* and *csdA*. The matching bases have been underlined. (Craig *et al.*, 1998 & Jiang *et al.*, 1996). In the *S. typhimurium cspA* and *cspB* sequences, the DNA sequence is given upstream of the transcriptional start site and the RNA sequence downstream.

The bases thought to be involved in RNase E cleavage are shown in circles on the *E. coli cspA* mRNA in figure 4.1 Comparison of the *S. typhimurium cspA* gene with that of *E. coli* shows that there are 3 mismatches in the 12 base motif, and in each case, an adenine is replaced by uracil, or *vice versa*. In addition, a guanosine has been inserted in the *S. typhimurium cspA* RNase E motif and such changes may alter the degradative effect of RNase E on *S. typhimurium cspA* RNA. Alternatively, the RNase E motif may differ slightly in *S. typhimurium*.

In this study, expression of *S. typhimurium cspA* was assessed by monitoring expression from a *cspA-lacZ* transcriptional fusion, where the *lacZ* gene was fused directly upstream of the translation initiation codon. Thus, regulatory elements, such as those in the promoter region, were maintained. Shifting a culture of exponentially growing EMG2 that had been transformed with pNJH6 from 37°C to 15°C, resulted in an 8-fold increase in the level of β-galactosidase activity after an incubation of 5 hours at 15°C. However, control studies that measured β-galactosidase from the *lac* operon present in EMG2 also showed a 5-fold increase in *lacZ* expression after 4 hours at 15°C, although expression decreased after incubation for 5 hours at 15°C (in the absence of IPTG). Taken together, at best, expression from the *cspA* promoter

only accounted for a 3-fold increase in *lacZ* expression over the native *lacZ* expression seen in EMG2. This is similar to the level of *cspA-lacZ* induction observed in the studies of *E. coli* by Goldenberg *et al.* (1996), where either transcriptional or translational *cspA-lacZ* fusions were incorporated on the chromosome (+81 and +346 bp from the transcriptional start site of *E. coli cspA*, respectively; the RBS is located at bases 146 to 150). It was proposed that *cspA-lacZ* expression required a *cis* acting element from the *cspA* gene, because these *cspA-lacZ* fusions were chromosomally integrated at the λ phage attachment site and β-galactosidase activity from the fusions was less than expected.

Similar results were found with a *cspB-lacZ* fusion from *B. subtilis*, where *lacZ* was fused to *cspB* immediately downstream of the RBS. Originally, chromosomal integration of the fusion occurred at the *B. subtilis* SPβ prophage site. When the culture was shifted to 10°C, no β-galactosidase activity was detected. However, when the *cspB-lacZ* fusion was transferred to the chromosome via a single cross-over homologous recombination event, β-galactosidase activity increase 6 to 8 fold over a 4 hour incubation at 10°C, following a shift from 37°C. In this situation the complete *B. subtilis cspB* locus was maintained in *cis*, immediately downstream of the *cspB-lacZ* fusion (Willimsky *et al.*, 1992). Reports of expression of the major cold shock genes of both *E. coli* and *B. subtilis* resulted in a level of β-galactosidase activity (generally around 100 Miller units) that was similar to that observed from *S. typhimurium cspA-lacZ*, from a low copy number plasmid. This suggests that the level of expression of *S. typhimurium cspA* was in line with that reported for *E. coli cspA* and *B. subtilis cspB*, although it is not possible to say that expression was entirely due to cold shock regulation of *cspA*.

The level of β-galactosidase cold shock induction detected from the *S. typhimurium cspA-lacZ* construct used for this study, was similar to the level of *lacZ* expression from EMG2. It is important to note that the *cspA* RBS was not present in this transcriptional fusion, and that the *lacZ* RBS was present. Thus, it is possible that the level of β-galactosidase activity was regulated, in part, by the presence of the RBS of

lacZ. This may account for the similar level of β-galactosidase activity from this construct and expression from *lacZ* in strain EMG2.

In this study, an appropriate host was used to keep the copy number of the plasmid containing the *S. typhimurium cspA-lacZ* construct, to a low level; a strategy also used for similar studies of *E. coli cspA* expression. In the *E. coli* studies, it was demonstrated that gene expression from the *cspA-lacZ* fusion increased 16-fold, following a temperature downshift to 15°C, which is almost double the level of expression observed for *S. typhimurium cspA* expression. However, the *E. coli cspA-lacZ* fusion used by Lee and colleagues (1994), contained the initial 10 codons of the ORF and included the putative DB. This evidence, together with the results for the *S. typhimurium cspA-lacZ* fusion in this chapter, supports the suggestion by Lee *et al.*, (1994) that the DB acts as a translational enhancer and that RNase E cleavage near the RBS is important for cold shock regulation.

Goldenberg *et al.* (1994) found that transcriptional fusion of *lacZ* to *cspA* (+81 bases from the transcriptional start site) increased the *cspA-lacZ* mRNA stability 18-fold at 37°C, and after 20 hours incubation at 20°C, the mRNA was completely stable for at least 60 minutes. Thus, it would be reasonable to suggest that the mRNA from the *S. typhimurium cspA-lacZ* construct in this chapter, was similarly stabilised. Extended expression of the *S. typhimurium cspB* gene has been observed when the gene was fused to a bioluminescent reporter element (Mu*dlux*), following a temperature shift from 30°C to 15°C. The level of *cspB* expression increased rapidly over the initial 2 hours, before decreasing slowly. Bioluminescence from the *cspB::*Mu*dlux* fusion was still detected after 15 hours at 15°C, which does not correlate with the level of CspB protein detected in 2D analysis of a culture that was similarly treated (described in chapter 6 of this thesis). The level of protein peaked after 1.5 hours and decreased after 4 hours at the low temperature. Thus it appeared that the addition of the Mu*dlux* element stabilised the fusion at 15°C.

Anomalous gene expression has also been reported for the *S. typhimurium proU* operon in the presence of a *lux* element (Forsberg *et al.*, 1994). The *proU* operon

encodes a high-affinity glycine betaine transport system that is induced following exposure to high osmolarity (Csonka & Hanson, 1991). Repression of *proU* in low osmolarity medium occurs via a downstream regulatory element that exists in the coding region of *proV* (the first member of the *proU* operon). Fusion of the *proU* promoter region, excluding this regulatory element, to *lacZ* was shown to lead to expression of *proU* under conditions of low osmolarity. However, when the *luxAB* genes were used as the reporter system, expression from the *proU* promoter was similar to that observed for the wild type, which suggested that the *luxAB* reporter system was able to substitute for the downstream regulatory element and repress *proU* expression in low osmolarity medium (Forsberg *et al.*, 1994).

Clearly, the differences in construction, chromosomal integration and copy number of the various *csp-lacZ* fusions of *S. typhimurium*, *E. coli* and *B. subtilis* have led to the dissimilar results described. The one, unambiguous conclusion is that gene expression from the various fusions does increase on incubation at sub-optimal temperatures. Perhaps in the light of the studies on the variety of regulatory features which affect *E. coli cspA*, the variations in gene expression are not surprising.

The use of a bioluminescent reporter system indicated that *S. typhimurium cspB* was cold shock inducible at temperatures just above and just below the minimum temperature for growth, and was not detected at 30°C. In addition, differences in the dynamics of *cspB* expression were observed when the cultures were in different growth phases, at the point of the temperature reduction. It should be noted that the *cspB*::Mu*dlux* reporter strain contained all the regulatory elements (putative and known) described previously for other *cspA* homologues, as the Mu*dlux* element was inserted 23 codons downstream of the translational start site of *cspB*. Thus, bioluminescence detected from this strain reflects translational expression of *cspB*. In contrast, expression from the *cspA-lacZ* reporter construct reflected transcriptional expression of *cspA* since *lacZ* was inserted upstream of the RBS of the *cspA* locus.

A temperature reduction from 30°C to 10°C resulted in a high level of expression from the *cspB::*Mu*dlux* fusion, when the culture was in exponential phase prior to the shift. Expression continued to increase for approximately 24 hours. In comparison to the reporter assays for expression of *cspA*, these results show that translational cold shock activation of *cspB* is substantially higher for *cspB* than for *cspA*.

Shifting the culture to 4°C resulted in a delayed increase in expression relative to that observed at 10°C. In addition, the level of expression was approximately 10-fold lower than that observed at 10°C. Thus, *S. typhimurium cspB* was shown to be cold shock inducible when the culture was in exponential phase, at both 10°C and 4°C. This study has demonstrated that *S. typhimurium cspB* is cold shock inducible at temperatures both above and below the minimum permissible for growth, when cells are in exponential phase. The decrease in the level of expression at 4°C relative to that at 10°C appeared to be similar to that observed in *E. coli* CspB protein levels. The level of induction of *E. coli* CspB was shown to be maximal after a shift from 37°C to 15°C and a shift to 6°C resulted in a level of induction of protein that was determined to be 5% that of the maximum level (Etchegaray *et al.*, 1996).

The effect of growth phase was investigated on the expression of *S. typhimurium cspB* at 10°C and 4°C. The levels of bioluminescence from the stationary phase cultures were approximately 10-fold and 100-fold lower, at 10°C and 4°C respectively, than for cultures that were exponentially growing. In addition, expression of *S. typhimurium cspB* from stationary phase cells was also delayed relative to that from exponentially growing cells. This data shows that expression of *S. typhimurium cspB* occurred at temperatures both above and below the minimum growth temperature, in both exponential and stationary phase. However, the level of expression appeared to be growth phase dependent since the level of bioluminescence at 4°C was very low. To our knowledge, this is the first report of expression of a cold shock homologue in both exponential and stationary phase, and both above and below the minimum growth temperature.

The results for *S. typhimurium cspB* complement those shown previously in our laboratory, that CspB is induced below a threshold temperature of 22°C (Craig *et al.*, 1998). Further studies have shown that CspA is also highly induced in *S. typhimurium* following a temperature decrease (this thesis, chapter 6). Therefore, the bioluminescence studies are also in agreement with several other reports, that frequently more than one member of the CspA-family is cold shock inducible. For example, CspA, CspB and CspG in *E. coli* (Lee *et al.*, 1994 & Nakashima *et al.*, 1996) and CspB, CspC and CspD in *B. subtilis* (Graumann *et al.*, 1996) are all cold shock inducible.

Previous studies of CspA and CspB from exponentially growing *E. coli* have shown both protein and mRNA were present at 10°C and 6°C, which are just above and just below the minimum temperature permissible for growth (Etchegaray *et al.*, 1996). In addition, Jeffereys and colleagues (1998) have shown that several proteins corresponding to the approximate size of a CspA-type protein were induced at 10°C and 5°C in an exponentially growing culture of *S. enteritidis*. It should be noted that this data has been gained from western hybridisation of proteins separated only by molecular weight and therefore does not distinguish between highly related proteins of a similar molecular weight, which may include members of the CspA family. Cold shock inducible members of the CspA family are thought to be involved in adapting bacteria to survive at sub-optimal temperatures. Our data shows that the adaptive process is initiated even at temperatures below the minimum growth temperature.

Reporter studies in *E. coli* have shown that CspD is expressed on entry to stationary phase, at 37°C (Yamanaka & Inouye, 1997). As yet, there are no reports of any members of the CspA family being cold shock inducible during stationary phase, although Jones and colleagues (1992) have shown that starvation of an exponentially growing culture for 4 hours at 10°C diminished induction of a large number of CspA homologues, including CspA. The bioluminescence data indicated that CspB was expressed in stationary phase following a reduction in temperature, albeit to a significantly lower level than observed for similar cultures in exponential phase.

Furthermore, there was a delay in CspB expression relative to exponentially growing cultures at the same temperature.

When bacterial cells enter stationary phase, a set of Pex proteins is induced specific to the conditions encountered. The alternative sigma factor, σ^S, encoded by *rpoS*, is a positive regulator of a large number of genes on entry to stationary phase. In addition, σ^S plays a regulatory role in several stress responses including oxidative shock, starvation, osmotic shock, heat shock and acid shock (Loewen & Hengge-Aronis, 1994, Loewen *et al.*, 1998). Moreover, σ^S has been shown to regulate stress response genes when the cells were in exponential phase (Hengge-Aronis, 1996). Studies with *E. coli*, using a *rpoS*::*lacZ* fusion have shown, that RpoS was induced 100-fold following a temperature shift from 42°C to 20°C, when the culture was in exponential phase (Sledjeski *et al.*, 1996).

The effect of σ^S was examined on *S. typhimurium cspB* cold inducible expression, using the *cspB*::Mu*dlux* fusion. When the cells were in exponential phase, σ^S did not appear to affect expression of *cspB*. However, when the cultures were grown to stationary phase, the level of *cspB* expression was lower in the absence of σ^S. At 10°C and 4°C, *cspB* expression was approximately 6-fold and 3-fold less, respectively, in the absence of σ^S. Thus σ^S appears to play a positive regulatory role in stationary phase expression of *cspB* both above and below the minimum temperature permissible for growth.

These results are in agreement with previous studies of genes affected by σ^S, although other factors are clearly involved in *cspB* regulation, since some expression of *cspB* was still detected in the absence of σ^S. Generally, σ^S has been shown to be a positive regulator of many genes that are induced in stationary phase (Loewen & Hengge-Aronis 1994). It is interesting that σ^S did not appear to play a regulatory role in *cspB* expression when the cultures were in exponential phase. This suggests that factors other than σ^S play a role in thermo-regulation of *cspB*, although it is possible that σ^S

is not cold shock inducible at low temperatures and therefore, is not able to regulate cold shock genes.

The regulatory effect of Fis on *S. typhimurium cspB* was also examined, using the *cspB::*Mu*dlux* fusion. As with the case for σ^S, Fis did not effect expression of *cspB* when the cultures were in exponential phase of growth. However, the effect of Fis on *cspB* expression when the cells were in stationary phase was very apparent. Expression of *cspB* was almost completely undetectable at both 10°C and 4°C, in the absence of Fis. Thus, Fis appeared to play a positive role in the regulation of *cspB* both above and below the minimum growth temperature. It should be noted, that the level of *cspB* expression at 4°C was particularly low even for *fis*$^+$ cells, therefore, these results should be viewed cautiously.

Fis has been shown to negatively regulate several genes, in a growth-phase dependent manner (Xu & Johnson, 1995). For example, reporter assays of transposon generated LacZ fusions, in the absence of Fis, showed that expression from some insertions in the *frg* locus increased in early exponential phase, while expression from other insertions in *frg* increased in mid and late exponential phase. Further insertions that were also growth phase dependent were mapped to genes that encode products required from growth under nutrient-poor conditions. In each of the transposon insertions investigated, Fis acted as a negative repressor. This is expected since Fis is induced to very high levels during lag phase, following a nutrient up-shift.

In the case of *S. typhimurium cspB*, Fis appeared to enhance expression, when the cells were in stationary phase, following temperature shifts to either 10°C or 4°C. This is interesting since it is known that the levels of Fis in stationary phase are relatively low (Ball *et al.*, 1992). However, Fis has previously been shown to effect gene expression of at least 3 genes (*frg*, *xylF* and *aldB*) when the cultures reached late exponential phase and stationary phase (Xu & Johnson, 1995). Thus it seems likely that Fis was also able to regulate *S. typhimurium cspB*, despite the low level of Fis protein. It is possible that expression of *S. typhimurium cspB* does not require

enhancement by Fis in exponential phase as other factors may be present. On entry into stationary phase, these factors may be absent which subsequently leads to requirement of Fis. The main factor that is known to affect *S. typhimurium cspB* expression is mRNA transcript stability (Craig *et al.*, 1998). Studies with *E. coli* have also shown that mRNA stability plays the major role in thermo-regulation of *cspA*. Other factors that have been shown to affect thermo-regulation of cold shock genes include the cold box region (Fang *et al.*, 1998), the Up element and the downstream box (Mitta *et al.*, 1997). However, these elements are intrinsic to the locus and would thus be expected to function regardless of the growth phase. (Studies of the regulatory elements have all taken place with cells that were exponentially growing). Thus, there may be other factors, such as proteins, which also play a role in thermo-regulation of cold shock genes.

In conclusion, reporter assays of transcriptional induction of *S. typhimurium cspA* was lower than predicted. However, this is probably due to the replacement of the downstream box, a region of DNA which may play an important role in translational enhancement. Furthermore, the plasmid based construct may have been lacking in *cis* acting element. Bioluminescent reporter studies have shown that *S. typhimurium* CspB is highly expressed following a downshift in temperature, and importantly, it is expressed both above and below the minimum temperature for growth. In addition, the results have indicated that Fis may play a role in enhancing CspB expression at 10°C and 4°C, during stationary phase. The alternative sigma factor, σ^s, may also play a role in stationary phase expression of CspB at 10°C, during stationary phase.

CHAPTER 5

SURVIVAL OF *S. typhimurium* AFTER RAPID CHILLING

5.1 INTRODUCTION

Pathogenic bacteria that are able to survive on foodstuffs have the potential to cause food poisoning. However, food-processing practices normally involve preventative measures to inhibit bacterial growth on raw meat carcasses. For example, *Salmonella* that are present on poultry carcasses must endure a rapid chilling step, which reduces the temperature of the carcasses to 4°C. Subsequent stages, such as food preservation and cooking are very effective in eliminating harmful bacteria if performed correctly. However, food poisoning may arise from cross contamination from an infected source to an uninfected source. For example, cross contamination can occur when raw meat or vegetables come into direct contact with pre-cooked food. In addition, inadequate storage can also result in growth of pathogens and increased numbers recovered from foods. Adaptive mechanisms enable bacteria to survive procedures such as low temperature storage, so that although growth cannot occur, the organisms enter stationary phase and are able to re-grow if the temperature changes to a permissible level. Such adaptation is thought to enhance the potential of harmful bacteria to cause food poisoning, following an unfortunately common error where pre-cooked food is stored above refrigerated temperatures.

Other factors such as the growth phase of the bacteria and the presence of other species of bacteria in foodstuffs are important features which may influence the survival of bacteria that encounter environmental stresses. The growth phase of bacteria has a large impact on bacterial survival following various stresses such as a change in temperature either above or below the optimum, or changes in osmolarity or oxidation. Numerous studies have shown that when bacteria are in the stationary phase a far greater proportion survive these types of stresses than those that are exponentially growing (see Kolter *et al.*, 1993). As early as 1958, Meynell showed that the survival of exponentially growing *E. coli* was more than three orders of magnitude lower than survival of stationary phase *E. coli*, following dilution of the cultures from 37°C into a solution at 4°C. In fact, almost 100% of the stationary phase culture survived the rapid cold shock (survival was measured in terms of the subsequent ability of *E. coli* to grow on nutrient agar plates). Furthermore, it has been

demonstrated that the dynamics of synthesis of the major cold shock protein of *S. typhimurium*, CspA, differ according to the degree of the temperature downshift and the bacterial growth phase (see chapter 6 of this thesis).

Several studies have been carried out to examine the effects of competition on survival of bacteria. Aldsworth *et al.*, (1998) have shown that when exponentially grown *S. typhimurium* were incubated with 10^8 cfu ml^{-1} of competitor bacteria, RpoS accumulated in significantly less time compared to competitor-free *S. typhimurium* cultures. In this case, RpoS accumulation was measured indirectly, by monitoring expression of an RpoS-dependent promoter, *spvRA*, that was fused to a reporter system, *luxAB*. Interestingly, this group have shown that survival of *S. typhimurium* increased from 0.2% to 60% following freeze stress, in the presence of 10^8 cfu ml^{-1} competitors (Aldsworth *et al.*, 1998).

5.1.1 The effects of sub-optimal temperatures on bacterial growth.

At optimal temperatures, bacterial growth rate is inversely proportional to the exponential of the absolute temperature, according to the Arrhenius relationship (Arrhenius, 1889). However, if the temperature is shifted either above or below the optimum range, the growth rate is less than predicted from the equation. To illustrate, the generation time of a culture of exponentially growing *E. coli* was found to increase from 30 minutes at 37°C to approximately 4.25 hours at 15°C (Broeze *et al.*, 1978).

5.1.1.i The lag phase.

In some bacteria, following a shift to low temperatures, a lag period occurs before growth resumes. The occurrence of the lag period appears to be dependent on factors such as the bacterial species, the media that the cells were grown in and the presence of amino acids in the media. The lag period has been demonstrated in bacteria such as *S. enteritidis* (Jeffreys *et al.*, 1998), *E. coli* (Jones *et al.*, 1992) and *Arthrobacter globiformis* (Berger *et al.*, 1996). Growth and division of *S. enteritidis* was shown to cease for 6 hours before resuming with a generation time of approximately 15 hours,

when a culture that was in early exponential phase was shifted from 37°C to 10°C. This culture was grown in rich media (LB) (Jeffreys *et al.*, 1998). Extensive analysis of growth at sub-optimal temperatures was carried out in the psychrotroph *A. globiformis*, in a liquid medium. Temperature shifts from 25°C to 15°C, 10°C or 4°C resulted in lag periods of 1 – 2 hours, 4 – 6 hours and 10 – 12 hours, respectively, before growth resumed. The respective generation times were 5.75 hours, 10.6 hours and 25 hours (Berger *et al.*, 1996). Earlier studies of exponentially growing *E. coli* cells, which were grown in glucose-MOPS medium, have shown that a 4 hour lag period occurred following a temperature decrease from 37°C to 10°C (Jones *et al.*, 1992). The lag time was dependent on the presence of amino acids in the medium, so that growth after the 4 hour lag only occurred when amino acids were added to the culture. It seemed that without the addition of amino acids, the culture would be maintained indefinitely in lag phase. The generation time following the temperature shift was approximately 24 hours. Interestingly, no such lag period was observed when *E. coli* cells that were grown in rich medium, were shifted from 37°C to either 25°C or 15°C. The respective generation times at the low temperatures were 1.5 hours and 4.2 hours (Broeze *et al.*, 1978).

Several studies have shown that a lag period in growth does not occur in other bacterial species, following a temperature reduction. Investigation into the effects of cold shock on several, commercially important strains of Lactic Acid bacteria have shown that there was no observable lag in growth of *Lactococcus lactis*, following a shift from 30°C to 10°C. The cultures were grown to exponential phase prior to the shift, in M17 medium supplemented with glucose (Kim & Dunn, 1997). In addition, studies with *B. subtilis* showed that there was no observable lag in growth following a shift from 37°C to 13°C, when the culture was grown exponentially prior to the shift, in rich medium. The generation time at this temperature was found to be approximately 25 hours. Interestingly, the minimum temperature permissible for growth was lower when the culture of *B. subtilis* was grown in rich medium as opposed to M9 medium, so that the respective temperatures were 8°C – 9°C or 12°C - 13°C (Graumann *et al.*, 1996). A less common food-borne pathogen, *Enterococcus*

faecalis is another example of a bacterium that does exhibit a lag period in growth following a cold shock. Studies where an exponentially growing culture of *E. faecalis* was shifted from 37°C to 8°C did not result in an observable growth lag. The generation time of the culture increased to 29 hours at 8°C (Panoff *et al.*, 1997).

In each of the above studies, the presence of putative cold-inducible members of the CspA family was revealed, either from *de novo* protein synthesis analysis or from reaction to CspA antibody.

5.1.1.ii The Stationary phase.

Studies with *E. coli* have shown that when a culture that was in stationary phase was rapidly chilled from 37°C to 4°C, almost 100% of the cells survived (measured in terms of colony forming ability). However, similarly chilling cells that were in early exponential phase led to a reduction in survival of three orders of magnitude (Meynell, 1958).

It has been known for some time that Gram negative bacteria that are in stationary phase are far more resistant to environmental stress than when in exponential phase of growth. Morphological changes, such as a reduction in cell volume is accompanied by cell wall changes and differences in protein expression. A specific set of Pex (post-exponential) proteins is induced, which is similar to many of the proteins induced during starvation. The stationary phase sigma subunit of RNA polymerase, σ^s, encoded by *rpoS*, is a major regulator of many genes expressed on entry into stationary phase. Other global regulators which play major roles in gene expression on entry to stationary phase include Lrp, the CRP-cAMP complex and IHF (for recent reviews see Kolter *et al.*, 1993, Siegle & Kolter, 1992, Loewen & Hengge-Aronis 1994).

5.1.2 Role of σ^S in stress protection.

The stationary phase sigma factor, σ^S has been implicated in the regulation of many stress responses, including the reaction to osmotic shock, oxidative shock and acid shock. Alteration of the sigma factor that binds to the core RNA polymerase and subsequently confers promoter specificity, is a common regulatory mechanism following environmental changes. The σ^S holoenzyme is required for expression of more than 30 *E. coli* genes in response to starvation and entry into stationary phase (Hengge-Aronis, 1993). This has resulted in its alternative designation, the general stress response sigma factor.

5.1.2.i The history of the genetic characterisation of RpoS.

The *rpoS* gene that encodes σ^S, was identified following several different studies of the *rpoS* allele that revealed quite different phenotypes. Due to these differences in apparent function, the gene was given a variety of names. Initial studies found that *E. coli rpoS*, then termed *nur*, enhanced resistance to near UV radiation. Importantly, *nur*- dependent resistance to near UV was growth phase dependent, so that resistance increased as the cells entered stationary phase and continued to increase into late stationary phase. Around the same time, studies with *E. coli* had shown that synthesis of the catalase hyperoxidase II (HPII), was dependent on *rpoS*, then termed *katF*. The *nur* gene was subsequently confirmed as an allele for *katF*. Shortly afterwards, an *E. coli* gene termed *appR* was found to required for the synthesis of acid phosphatase from *appA*, and was also mapped to the *katF* locus. Studies of carbon starvation-inducible genes in *E. coli* showed that mutagenesis of *rpoS*, then termed *csi2*, resulted in lack of resistance to hydrogen peroxide, lack of thermotolerence and lack of *appA* expression. These studies strongly suggested that *rpoS* affected a diverse group of cellular functions and encoded a regulatory gene controlling a significant number of genes involved in the stationary phase response (for a review see Loewen & Hengge-Aronis, 1994).

Characterisation of the *katF* gene product, lead to identification of a protein that was closely related to the σ^{70} family of transcription factors. Following genetic and phenotypic characterisation, *katF* was renamed *rpoS* and its gene product was termed σ^{s}. Members of the σ^{70} family are categorised into three groups. Group 1 contains the primary sigma factors, which are essential for cell survival and responsible for the majority of RNA synthesis in exponentially growing cells. The amino acid sequence identity of the sigma factors in this group is at least 51%. Group 2 contains *E. coli* σ^{s} and the *Streptomyces coelicolor* alternative sigma factors. These sigma factors are not essential for growth of exponential phase cells. It has been suggested that group 2 sigma factors recognise similar promoter sequences to group 1 sigma factors. Group 3 contains the remaining sigma factors that are quite divergent from the group 1 sigmas. It appears that these sigmas fall into functional subgroups that include members from diverse organisms. For example, the *E. coli* heat shock sigma (σ^{H}) is 94% identical (in terms of the amino acid sequence) to its counterpart in *Citrobacter freundii*. However, *E. coli* σ^{H} shares only 24% identity to *E. coli* σ^{70} (Lonetto *et al.*, 1992). It has been proposed that σ^{s} recognises a promoter sequence consisting of the –10 sequence together with an upstream region of intrinsic DNA curvature (Espinosa-Urgel *et al.*, 1996).

The role of σ^{s} has been examined in the acid tolerance response of *S. typhimurium*. Two distinct mechanisms exist that allow *S. typhimurium* to tolerate exposure to an acidic environment and these differ in the growth phase at which activation occurs. The acid-dependent response is transient and requires pre-adaptive exposure to a moderately low pH, which protects the bacteria against more severe acid challenges. Analysis of the acid tolerance response (at 37°C) of exponentially growing cells, have shown that initially, the response is RpoS independent. However, after approximately 20 minutes RpoS is required for sustained tolerance. Indeed, RpoS was observed to increase approximately 4-fold in exponentially growing cells that were exposed to pH 4.4 for 60 minutes (Lee *et al.*, 1995). The second mechanism of acid resistance was exhibited by cells that were in stationary phase, and consequently attained resistance to a similar decrease in pH from 7.7 to 4.3.

Studies of the osmotic stress response of *E. coli* have shown that at least 16 σ^s-controlled proteins that were expressed in stationary phase were also induced following an osmotic up-shift, as identified by 2-D PAGE. Moreover, the level of σ^s in osmotically challenged (0.3 M NaCl) cells that were in exponential phase, was similar to the level observed in untreated cells that were in stationary phase. The same effect was also observed in the presence of sucrose, which confirmed that the level of σ^s increased in a true osmoregulatory manner rather than just due to the salt effect (Muffler *et al.*, 1996).

5.1.2.ii RpoS expression at low temperatures.

Studies with *E. coli* have shown that σ^s is also induced in exponential phase cells following a decrease in temperature. Determination of *E. coli* RpoS expression at 20°C was examined using a *rpoS::lacZ* translational fusion. β-galactosidase activity was observed to increase more than 100-fold following a shift of an exponentially growing culture from 30°C to 20°C. Furthermore, the increase in RpoS production was dependent on the small DsrA untranslated RNA, which affects gene expression by antagonising H-NS-mediated transcriptional silencing (Sledjeski *et al.*, 1996). Indeed, previous studies in *E. coli* have shown that H-NS represses RpoS production in exponentially growing cells at both 30°C and 37°C. Analysis of *rpoS* mRNA and RpoS protein indicated that H-NS acted either on the translation of *rpoS* mRNA or on the stability of RpoS (Yamashino *et al.*, 1995).

5.1.3 Cross protection to environmental stresses.

A large body of work that has examined cross protection between different environmental stresses. Exposure to a sub-lethal environmental stress has been shown to provide a degree of protection to a different stress. Starvation is known to increase resistance of bacterial cultures to many environmental stresses, such as heat shock, osmotic shock and oxidative shock (Jenkins *et al.*, 1988, Jenkins *et al.*, 1990). Studies on glucose starvation in *E. coli* have shown that starved cells survived a

temperature shift from 37°C to 57°C markedly better than a similar culture in exponential phase of growth. In fact, after 6 minutes at 57°C, the exponentially growing cells had lost viability (as measured by colony formation ability), while the culture that had been glucose starved for 4 hours were 95 % viable. Similar experiments where the culture was starved of glucose for 4 hours also provided greater than 95 % protection to 15 mM H_2O_2. In contrast, a culture that was grown at 29°C, to mid-exponential phase, treated with 15 mM H_2O_2 and subsequently shifted to 57°C, was found to be more sensitive to the heat shock than untreated cells (Jenkins *et al.*, 1988). This is contrary to other reports, although, the heat challenge was to a higher temperature and for a longer duration, than that reported by other studies. This may account for the differences observed.

Two-dimensional (2-D) analysis of stress-induced proteins of *S. typhimurium* indicated that the same set of proteins was consistently induced by a variety of starvation conditions. However, there was little overlap between the starvation inducible proteins and those induced either by heat shock or anaerobic shock. The nutrient limitations included phosphate, nicotinate, ammonium, and glucose starvation, while the heat stress involved a shift from 30°C to 45°C and oxygen was excluded from the culture by overlaying with sterile paraffin oil. In each case, the culture was in exponential growth prior to the stress imposition (Spector *et al.*, 1986).

Studies with *B. subtilis* have also shown a degree of cross protection arising from osmotic shock and heat shock, as well as pre-adaptation to an osmotic or heat shock. Addition of 2 % (w/v) NaCl to a *B. subtilis* culture for 30 minutes, pre-adapted the culture to an otherwise lethal concentration of 6 % (w/v) NaCl. Survival of the pre-adapted culture was almost 100%. In comparison, survival of the culture that had not been pre-treated was less than 10% and 2-D PAGE analysis showed that treatment with 2 % NaCl induced stress proteins. A similar type of protection was also observed with heat shock. Survival of cultures that were incubated at 48°C for 30 minutes prior to incubation at 52°C, was also close to 100%. In comparison, survival was less than 1% for cultures that had not been pre-adapted. The heat shock proteins,

DnaK and GroEL, were induced during the pre-adaptation period (as shown by 2D-gel electrophoresis). The pre-adaptive heat stress at 48°C for 30 minutes also conferred 100 % survival when 6 % NaCl was added to the culture. However, pre-adaptive salt stress of 4 % NaCl for 30 minutes at 37°C only provided approximately 40 % survival when the culture was shifted to 52°C (Volker *et al.*, 1992). 2-D PAGE analysis showed that 5 of the proteins induced during osmotic shock corresponded to 5 starvation Pex (post-exponential) proteins (Jenkins *et al.*, 1990).

Studies on survival of *E. coli* following a rapid cold shock to 4°C, have shown that addition of an osmoprotectant protects the cells that were in exponential phase of growth, during the cold shock. When samples of the culture were diluted into a solution at 4°C that contained 0.3 M sucrose, complete survival of the culture was observed. In contrast, survival of the exponentially growing culture that was diluted into the chilled solution without added sucrose, was 3 orders of magnitude lower (Meynell, 1958).

5.1.3.i Pre-adaptation to the oxidative stress response in *Salmonella typhimurium.*

Studies by Mackey and Derrick (1986) have shown that exposure to sub-lethal concentrations of hydrogen peroxide pre-adapted culture of *S. typhimurium* to severe oxidative stress. The viability of *S. typhimurium* that was grown in M9 medium at 20°C, decreased approximately 1.5 orders of magnitude when 30 μM H_2O_2 was added to the culture. In contrast, viability was complete in a similar culture that was grown in rich medium. Further analysis showed that peroxide levels in rich medium ranged from 12 – 30 μM, while M9 contained no peroxide. Thus, the presence of a non-lethal level of peroxide in the medium appeared to protect the culture against further peroxide exposure.

5.1.3.ii The universal stress protein, UspA.

Studies of a small, 13.5 KDa, cytoplasmic *E. coli* protein, termed UspA (the universal stress protein), indicate that it is induced by a variety of stresses. The UspA protein was detected on 2-D PAGE gels following glucose starvation, phosphate starvation, isoleucine starvation, addition of serine hydroxamate, addition of cadmium chloride, osmotic stress and heat shock (Nystrom & Neidhart, 1992). Artificial overproduction of UspA, by 10-fold, lead to a 5-fold reduction in the growth rate of *E. coli* cells grown in minimal glucose medium. In contrast, a similar increase in UspA levels did not affect the growth rate of cells that were grown in either minimal medium supplemented with amino acids, or in LB medium. An increase of UspA also lead to a 6-fold increase in the lag period, following glucose starvation for 24 hours and the subsequent addition of glucose. The increase in the lag period was shortened from 24 hours to 12 hours when amino acids were added to the medium. In addition, the expression of some proteins increased, including MetE, OmpA, OmpF, CirA, FepA, Mdh and GltA. Interestingly, the pI of 6 proteins was altered, following over-expression of UspA. It has been proposed, therefore, that UspA may be involved in post-translational modification of proteins during growth arrest, in particular, in kinase activities (Nystrom & Neidhardt, 1996)

5.1.4 The viable but non-culturable phenomenon.

Studies of cell growth and survival at sub-optimal temperatures routinely use cell plating and optical density as methods for cell measurements. However, these methods fail to detect bacteria that may be in an altered state of viability where they exhibit an inability to form colonies on solid agar. Caution must therefore be used when interpreting such assays. Bacteria that cause food-associated illness such as *S. typhimurium*, *E. coli*, *Pseudomonas*, *Campylobacter jejuni* and *Vibrio vulnificus* have been observed to enter the so called viable-but-non-culturable (VBNC) state (Chmielewski & Frank, 1995; Smith *et al.*, 1994). In addition, previous studies of bacterial survival in marine environments have demonstrated that cold shock,

starvation and osmotic shock, which result in sub-lethal injury, can induce the VBNC state in selected organisms (Smith *et al.*, 1994).

In particular, there are a number of studies that show that *Salmonella* subspecies can be induced to enter a viable but non-culturable (VBNC) state and, importantly, culturability can be recovered from this state. Normally, a combination of low temperature and starvation are used to induced the VBNC state and these conditions also mimic those conditions that environmental bacteria are likely to encounter. *Salmonella typhimurium* has been shown to reach a non-culturable state, on nutrient agar plates, following starvation in sterile seawater for 35 days (Lebaron & Joux, 1994). In comparison, the culturability of *Alteromonas haloplanktis*, an indigenous marine species, decreased only 2 orders of magnitude over the same period. However, addition of 50 mg l^{-1} of peptone resulted in recovery of culturability of approximately 60 % of the original *S. typhimurium* population, after 60 hours of incubation. Viability of the cultures was measured by analysing the chromosome content of the cells, which suggested that there was little change of the direct viable count and that cell lysis was minimal during the period of starvation (Lebaron & Joux, 1994).

Further studies have shown the temperature plays an important role in the induction of the VBNC state of *Salmonella*. Studies with *S. enteritidis* showed that during a 10 week period, culturability decreased by 90 % at 21°C, when a culture was incubated in 7.35 mM phosphate. However, culturability ceased after 5 weeks when the culture was incubated in a similar solution at 7°C (Chmielewski & Frank, 1995). (It should be noted that this temperature is very close to the minimum reported growth temperature in nutrient rich media for *S. enteritidis* (Mossel *et al.*, 1981)). In a separate study, the response to polar seawater was investigated in *S. typhimurium* (Smith *et al.*, 1994). Cultures that were grown to early stationary phase, at 37°C, were inoculated in sterile seawater and incubated at in a polar marine environment –1.8°C. Colony forming ability, measured on tryptone agar plates at 25°C, decreased rapidly and after 25 hours was approximately 4 orders of magnitude less than the direct

viable count (DVC). Notably, the DVC had decreased by less than 1 order of magnitude relative to the original value, during this time period. (The DVC was measured by the ability to grow in the presence of naladixic acid).

It is generally thought that the VBNC state is a progressive growth state that occurs after the bacteria have entered stationary phase. Differences in the detection of viable organisms and methods of cell recovery from the VBNC state mean that the basis for this phenomenon remains unresolved.

5.1.4.i Methods used for detecting the VBNC state.

The conventional method for detecting bacteria in the VBNC state was developed by Kogure *et al.*, (1978). In this test, cell viability direct viable count (DVC) of Gram negative bacteria is defined as the ability to elongate in the presence of naladixic acid, and is visualised by staining with acridine orange. A moderate concentration of naladixic acid (0.002 %) specifically inhibits bacterial DNA synthesis, without affecting other essential metabolic activities of the cell. Thus, in the presence of naladixic acid and suitable nutrients, viable bacteria continue to grow without dividing, which leads to the formation of elongated, filamentous cells. In addition, metabolically active cells can be seen to fluoresce reddish-orange in the presence of acridine orange, which is used to stain the cells in combination with the naladixic acid treatment. In contrast, inactive bacteria, which contain a low level of cellular RNA, fluoresce greenish-white (Kogure *et al.*, 1978).

Alternative methods which are used to assess cell viability utilise dyes that indicate the occurrence of respiration. For example, the electron transport of a small aliquot of a bacterial culture can be measured by addition of 5-cyano-2,3-ditoyl tetrazolium chloride (CTC). Tetrazolium reduction indicates active respiration, and after chromosomal DNA staining with 4',6-diamidino-2-phenylindole (DAPI), active cells fluoresce (Rodriguez *et al.*, 1992). In conjunction with these approaches, detection of

specific bacterial species is also frequently confirmed using fluorescently labelled antibodies that bind to cell wall components.

5.1.4.ii Culturability at low temperatures and at different osmotic pressures.

Survival studies of *S. enteritidis* have shown that cells enter a VBNC state following starvation at 7°C, in 7.35 mM phosphate buffer. Culturability was lost after 5 weeks although DVC measurements showed that the population was maintained at 10^4 cell ml^{-1} (Chmeielewski & Frank, 1995). Further studies were carried out at 21°C, and in this case, culturability was found to decrease by more than 90 % after 10 weeks, in agreement with DVC measurements. The experiments were repeated, at both 7°C and 21°C in saline solution with NaCl concentration at 17 mM, 1.7 mM or 0.17 mM. However, no differences were observed between culturability and DVC under the different osmotic conditions (Chmeielewski & Frank, 1995).

Studies of *S. typhimurium*, *E. coli* and *Y. enterocolitica* incubated in aged, sterile seawater for 54 days at –1.8 °C, have shown that all these bacteria enter into VBNC states (Smith *et al.*, 1994). For *S. typhimurium*, colony forming ability on TLY and TLYD agar declined by 5 orders of magnitude, while the DVC count remained within 79 % of the original CFU count, during this period (Smith *et al.*, 1994). (Colony forming ability and determination of DVC were carried out at 25°C). Similar results were obtained for *E. coli* and *Y. enterocolitica*. In general, after 40 days in aged, sterile seawater at –1.8°C, the CFU count was between 5 and 6 orders of magnitude lower than the DVC for all three species (Smith *et al.*, 1994).

5.1.4.iii The role of σ^s in VBNC.

The role of σ^s was examined in culturability of *S. typhimurium* and *E. coli* that were transferred to sterile, filtered seawater, at room temperature. The bacteria were grown either to exponential phase or to stationary phase, prior to inoculation into seawater

at high or low osmolarity. In comparison to a wild type parental strain, deficiency in *rpoS* in a low osmolarity environment led to a reduction of 3 orders of magnitude in the ratio of culturable cells to viable cells, when the cells were in stationary phase. A similar, but slightly diminished effect was observed in a high osmolarity environment. When the cells were exponentially growing prior to the shift, the ratio of culturable to viable cells also decreased more than 2 orders of magnitude in the *rpoS* mutated strain, in high osmolarity seawater. However, no difference was observed for the *rpoS* mutated strain and the wild type strain in low osmolarity seawater (Munro *et al.*, 1995). Thus, these results indicate that RpoS plays a major role in adapting bacteria to high osmotic environments, even when exponentially growing.

5.2 RESULTS

The effect of rapid chilling on *S. typhimurium* was examined in order to determine its effect on the survival of the culture when applied at different phases of growth. In addition, the role of σ^s in survival at 4°C was examined in a strain that had a mutated *rpoS* gene. Furthermore, in light of the cross protection studies, in particular by Meynell (1958), the effects of 0.3 M sucrose in the diluent were also examined.

5.2.1 The effect of rapid chilling on *S. typhimurium*.

To assess the effect of rapid chilling on *S. typhimurium*, a similar method was used to the one described in Meynell (1958). In short, an overnight culture of SL1344, grown in NB with shaking was diluted 1000–fold into NB and grown at 37°C for 5 hours, with shaking. Two sets of parallel samples were subsequently taken at 30 minute intervals. One set was diluted 10–fold into 25% Ringer's solution at 37°C and plated on NB agar immediately. The second set was diluted 10-fold into 25 % Ringer's at 4°C and incubated at this temperature for 2 hours prior to plating on NB agar, in triplicate. Colonies were allowed to form on NB agar at 37°C for approximately 18 hours. Survival was assessed by the number of CFU after chilling divided by the number of CFU prior to chilling, expressed as a percentage. In light of the on-going discussions on viability and culturability, it is important to note that in these chilling assays, survival refers to the ability to form colonies on solid agar.

Table 5.1 shows the numbers of CFU obtained from both the chilled and unchilled samples over 5 hours. In addition, survival at 4°C is also shown. When displayed graphically (figure 5.1), it can be seen that the number of CFU produced by SL1344 at 37°C increased with time, producing a characteristic growth curve, which included a lag phase, exponential growth period and early stationary phase period. The parallel set of chilled samples (at 4°) showed the number of CFU recovered was very similar during the lag period and in early stationary phases of growth. However, during exponential phase, and in particular in early exponential phase, there was a

substantial decrease in survival. In fact, when diluted into cold Ringer's solution after 150 minutes of growth, the number of CFU of the chilled samples was only 4 % that of the unchilled samples.

Time (minutes)	CFU from 37°C samples	CFU from 4°C samples	Survival (%)
0	6.091 (+/- 0.120)	6.122 (+/- 0.250)	101
30	6.090 (+/- 0.151)	6.061 (+/- 0.190)	96
60	6.279 (+/- 0.00)	6.122 (+/- 0.109)	70
90	6.502 (+/- 0.024)	6.327 (+/- 0.015)	67
120	6.821 (+/- 0.012)	6.423 (+/- 0.025)	40
150	7.237 (+/- 0.031)	5.875 (+/- 0.000)	4
180	7.544 (+/- 0.301)	6.380 (+/- 0.044)	7
210	7.975 (+/- 0.025)	7.332 (+/- 0.136)	23
240	8.394 (+/- 0.100)	8.352 (+/- 0.038)	91
270	8.690 (+/- 0.005)	8.568 (+/- 0.021)	76
300	8.975 (+/- 0.065)	8.873 (+/- 0.057)	79

Table 5.1 The effect of rapidly chilling *S. typhimurium* to 4°C. An overnight culture of SL1344 was diluted 1000 fold into NB and incubated at 37°C for 5 hours, with shaking. Two sets of samples were then taken at 30 minute intervals and diluted into 25 % Ringer's solution at either 37°C or 4°C. The colony forming ability was measured after 2 hours at 4°C. Survival is expressed determined as a percentage of the CFU at 37°. The standard deviation of the mean is given in parenthesis.

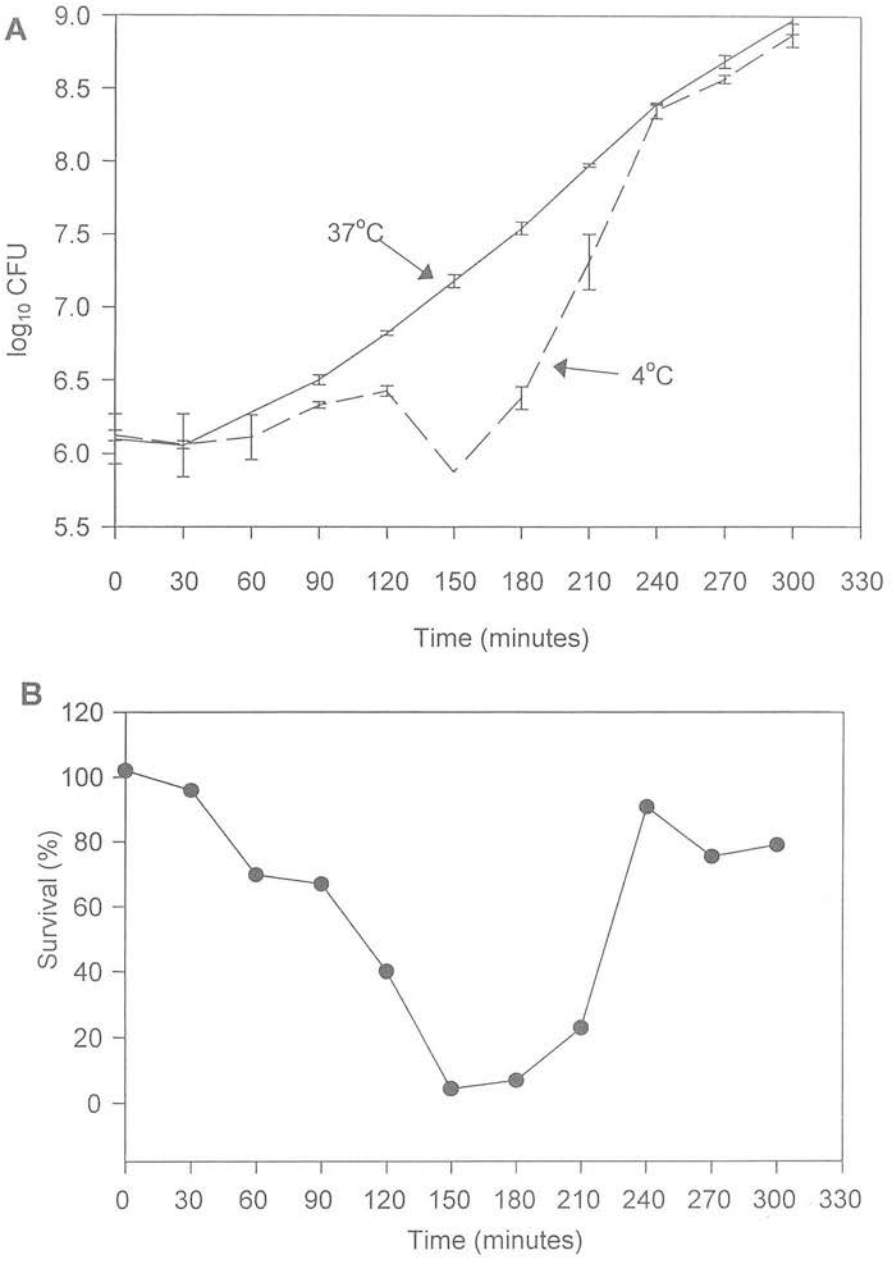


Figure 5.1 The effect of rapidly chilling a culture of *S. typhimurium* from 37°C to 4°C.

An overnight culture of SL1344 was diluted 1000 fold into NB and incubated at 37°C for 5 hours, with shaking. Two sets of samples were taken at 30 minute intervals during this time, and diluted in 25 % Ringer's either at 37°C (solid line) or at 4°C (small dashed line). The samples were plated immediately (37°C) or after incubation at 4°C for 2 hours, respectively (panel **A**). Survival at 4°C was determined as a percentage of the CFU at 37°C, (panel **B**).

5.2.2 The role of osmoprotectants in rapid chilling of *S. typhimurium*.

In a previous study Meynell (1935) found that survival of exponentially growing *E. coli* was unaltered by rapid chilling to 4°C when an osmoprotectant was included in the diluent. Thus, the effect of the addition of 0.3 M sucrose on survival at low temperature was also investigated, for SL1344. Bacteria were diluted into 25% Ringer's solution at either 37°C or 4°C, as described above. In addition, a third set of samples was diluted in 25% Ringer's solution that contained 0.3 M sucrose, at 4°C. The number of CFU obtained from the unchilled samples (37°C) and the chilled samples (4°C), with and without 0.3 M sucrose are shown in table 5.2 and figure 5.2.

Time (minutes)	CFU from 37°C samples	CFU from 4°C samples	Survival (%)	CFU from 4°C samples +0.3 M sucrose	Survival (%) + 0.3 M sucrose
0	6.455 (+/- 0.022)	6.421 (+/- 0.070)	93	6.418 (+/- 0.093)	93
30	6.380 (+/- 0.00)	6.410 (+/- 0.042)	107	6.385 (+/- 0.032)	101
60	6.457 (+/- 0.048)	6.302 (+/- 0.017)	70	6.407 (+/- 0.078)	90
90	6.634 (+/- 0.132)	5.459 (+/- 0.005)	6	6.444 (+/- 0.115)	63
120	7.034 (+/- 0.01)	5.316 (+/- 0.197)	2	5.953 (+/- 0.036)	8
150	7.375 (+/- 0.046)	5.851 (+/- 0.00)	3	6.022 (+/- 0.049)	4
180	7.877 (+/- 0.081)	6.151 (+/- 0.023)	2	6.159 (+/- 0.158)	2
210	8.197 (+/- 0.010)	6.976 (+/- 0.216)	6	7.849 (+/- 0.037)	45
240	8.619 (+/- 0.063)	8.531 (+/- 0.032)	81	8.620 (+/- 0.025)	100
270	8.876 (+/- 0.018)	8.902 (+/- 0.002)	106	8.877 (+/- 0.049)	101
300	9.034 9 (+/- 0.079)	9.032 (+/- 0.032)	99	9.032 (+/- 0.060)	99

Table 5.2 The effect of chilling *S. typhimurium* to 4°C, in the presence of an osmoprotectant. A culture of SL1344 was prepared as described in the legend for table 5.1. Subsequently, three sets of samples were taken at 30 minute intervals and diluted into 25 % Ringer's solution at 37°C, or at 4°C, with or without 0.3 M sucrose. The colony forming ability was measured and survival at 4°C was determined as a percentage of the CFU at 37°C. The samples were plated in triplicate and standard deviation of the mean is shown in parenthesis.

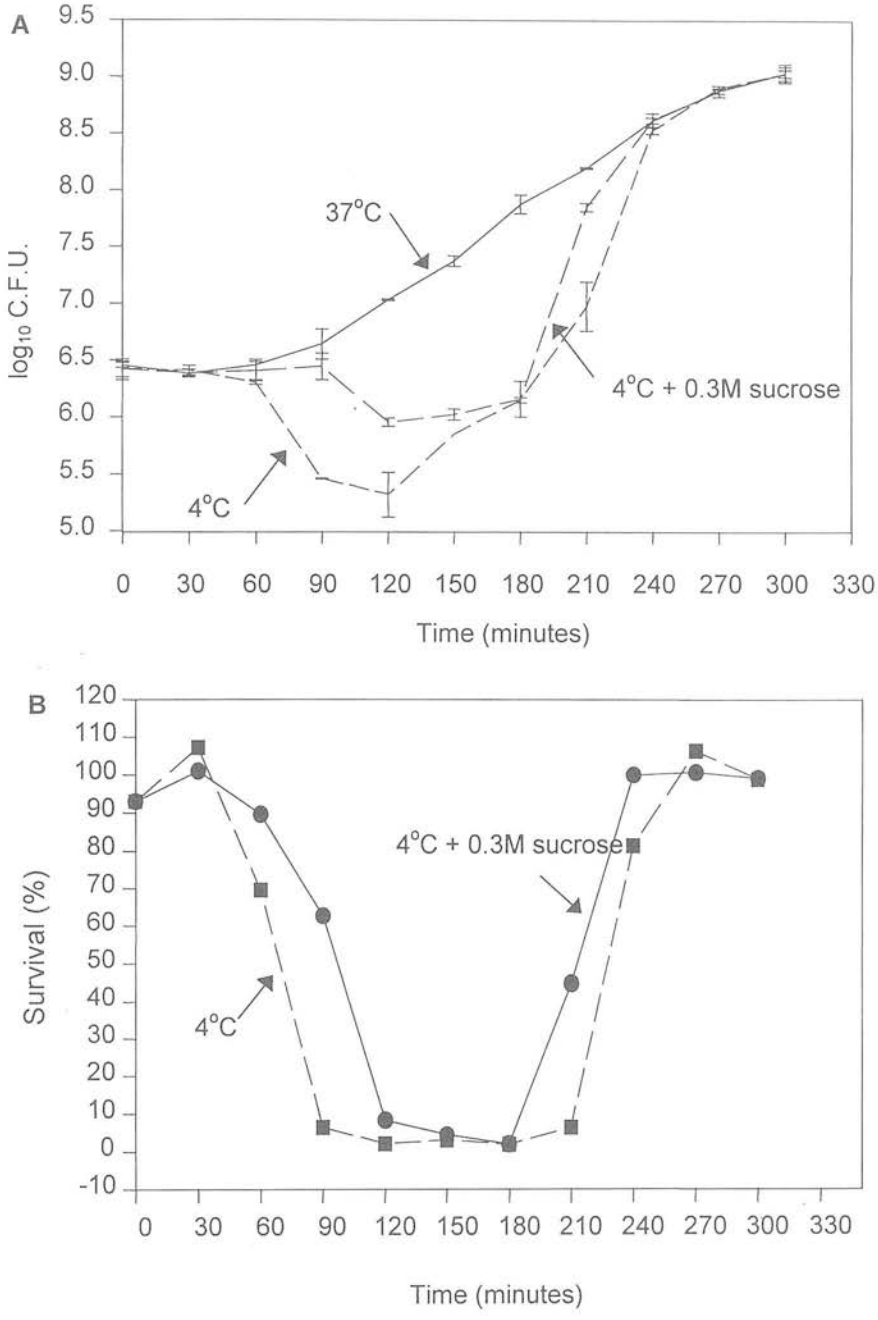


Figure 5.2 The effect of rapidly chilling *S. typhimurium* in the presence or absence of an osmoprotectant.

The culture of SL1344 was prepared as described in the legend for Figure 5.1. Subsequently, 3 sets of samples were taken at 30 minute intervals and diluted in 25 % Ringer's either at 37°C (solid line) or at 4°C with 0.3 M sucrose (large dashed line) or without 0.3 M sucrose (small dashed line). The samples were plated immediately (37°C) or after incubation at 4°C for 2 hours, (panel **A**). Survival at 4°C was determined as a percentage of the CFU at 37°C, survival at 4°C with 0.3 M sucrose is indicated by filled circles and survival without added sucrose is indicated by filled squares (panel **B**).

The number of CFU of the chilled samples without added sucrose decreased in early exponential phase, in the manner seen before (figure 5.1). However, addition of 0.3 M sucrose to the diluent increased survival, especially of the samples that were incubated for 90 minutes or 210 minutes at 37°C, prior to chilling. The protective effect of added sucrose was limited since the number of CFU of the chilled samples that were taken from the culture after 150 minutes and 180 minutes, were approximately the same.

5.2.3 The role of σ^S in rapid chilling of *S. typhimurium.*

The rapid chilling assays have shown that when cells are in stationary phase, survival is unaffected following chilling at 4°C. In addition, studies with *E. coli* have shown that an *rpoS-lacZ* fusion was highly induced when exponentially growing cells are shifted from 37°C to 20°C (Sledjeski *et al.*, 1996). Therefore, the role of the stress sigma factor, σ^S, was examined in the rapid chilling assays. The assays were carried out as described previously at 37°C and 4°C with MPG480 (*rpoS* was inactivated by insertion of *bla*, and the strain used was a SL1344 derivative). The number of CFU obtained for the chilled (4°C) and unchilled (37°C) samples and percentage of survival at 4°C are shown in table 5.3 and graphically in figure 5.3.

The pattern of survival of MPG480 at 4°C was very similar to survival of SL1344 at 4°C, when the cultures were in exponential phase. However, when the culture of MPG480 entered stationary phase, survival did not increase to the same extent as observed in a similar culture of SL1344, although survival did increase compared to that of cells that were in exponential phase. Survival of the samples taken from the culture of MPG480 after 300 minutes was 64 % at 4°C, whereas, samples taken from the culture of SL1344 was 79%. In the lag period, survival of MPG480 was marginally less than that observed for SL1344, and survival was approximately the same when either strain was in early exponential phase prior to the cold shock.

Time (minutes)	CFU from 37°C samples	CFU from 4°C samples	Survival (%)
0	6.145 (+/- 0.032)	6.119 (+/- 0.010)	94
30	6.132 (+/- 0.035)	6.056 (+/- 0.014)	84
60	6.284 (+/- 0.074)	6.112 (+/- 0.041)	69
90	6.439 (+/- 0.064)	6.312 (+/- 0.053)	75
120	6.670 (+/- 0.035)	6.332 (+/- 0.000)	45
150	6.988 (+/- 0.003)	6.111 (+/- 0.015)	13
180	7.362 (+/- 0.067)	6.183 (+/- 0.036)	6
210	7.708 (+/- 0.100)	6.824 (+/- 0.015)	13
240	8.095 (+/- 0.005)	7.834 (+/- 0.143)	55
270	8.528 (+/- 0.081)	8.312 (+/- 0.097)	74
300	8.778 (+/- 0.022)	8.585 (+/- 0.097)	64

Table 5.3 The effect of rapidly chilling *S. typhimurium* MPG480 (*rpoS*) to 4°C. A culture of MPG480 was treated as described in the legend for table 5.1. Subsequently, 2 sets of samples were taken at 30 minute intervals and diluted into 25 % Ringer's solution at 37°C, or at 4°C. The colony forming ability was measured and survival at 4°C was determined as a percentage of the CFU at 37°C. The samples were plated in triplicate and the standard deviation of the mean is shown in parenthesis.

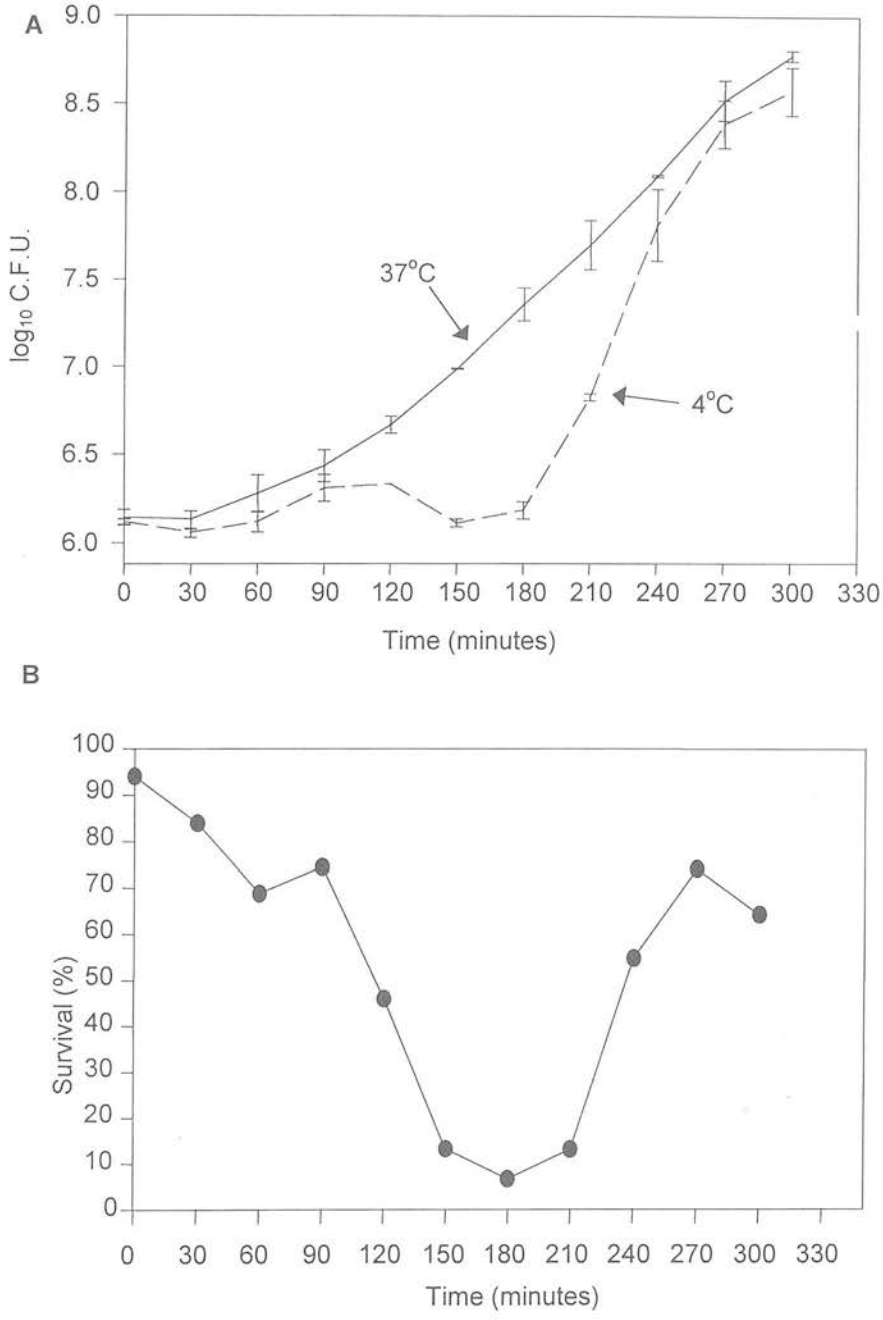


Figure 5.3 The effect of chilling a *S. typhimurium rpoS* derivative (MPG480).

A culture of MPG480 was prepared as described in the legend for figure 5.1. Subsequently 2 sets of samples were taken at 30 minute intervals and diluted in 25 % Ringer's either at 37°C (solid line) or at 4°C (small dashed line). The samples were plated immediately (37°C) or after incubation at 4°C for 2 hours, respectively, (panel **A**). Survival at 4°C was determined as a percentage of the CFU at 37°C, (panel **B**).

5.3 Discussion

Many studies have shown that following exposure to environmental challenges, survival of bacteria is growth-phase dependent (for a review see Kolter *et al.*, 1993). In addition, in some circumstances, it has been demonstrated that pre-adaptation to stresses, such as heat shock, greatly increases survival to a later, more severe shock (Jenkins *et al.*, 1988). The role of the alternative sigma factor, σ^S, has been shown to be important in protecting bacteria against environmental challenges. However, such protection is not straight forward, requiring different genes to be induced under different circumstances, for example, during the mechanisms of acid tolerance response and osmotic shock response of *S. typhimurium*. The survival of *S. typhimurium* has been investigated following a rapid decrease in temperature, from 37°C to 4°C. The effect of growth phase and the presence of an osmolyte have also been examined. In addition, the role of σ^S in survival at low temperatures was investigated.

It is well known that Gram negative bacteria can exhibit the same degree of survival when they are in stationary phase, as that observed in sporulating bacteria. Many of the genes that are expressed on entry into stationary phase are regulated by the sigma factor, σ^S (Kolter *et al.*, 1993). In fact, σ^S has also been shown to be responsible for expression of stress response genes in exponentially growing cells (Hengge-Aronis, 1996). However, the complexity of induction of such genes is illustrated well in the acid tolerance response. There are at least two mechanisms of response for *S. typhimurium* (Lee *et al.*, 1995), and three mechanisms have been identified in *E. coli* (Castanie-Cornet *et al.*, 1999). For *S. typhimurium*, the acid-dependent response is induced after pre-exposure to a moderately low pH, which then protects the cells against a more severe challenge. This response is transient and requires RpoS expression after approximately 20 minutes to elicit sustained acid tolerance. The alternative mechanism was found to be activated when the cells were in stationary phase (Lee *et al.*, 1995). The acid tolerance response of *E. coli* involves three defined systems that can protect the cells against acid stress as low as pH 2.0 to 2.5. The first is a glucose-repressed system induced in LB that is dependent on σ^S. The two other

mechanisms require glutamic acid during the acid stress, and arginine together with an inducible arginine decarboxylase, respectively. All three mechanisms have been observed in stationary phase cells (Castanie-Cornet *et al.*, 1999). Thus, growth phase and the role of σ^s clearly play important roles in stress responses, such as the acid tolerance response.

In this study, survival of *S. typhimurium* at 4°C has been shown to be growth phase-dependent. Cells that were in stationary phase and lag phase cultures were completely resistant to sudden cold shock. However, survival at 4°C decreased to 4% in early exponentially phase, although survived increased to 79 % in late exponential phase, after 5 hours of growth at 37°C. The trend from this data is in line with previous observations for rapid chilling of *E. coli* (Meynell, 1938), although survival of *E. coli* was observed to decrease by 4 orders of magnitude in early exponential phase (as opposed to 25-fold for *S. typhimurium* in the present study). It is possible that the observed differences in the degree of survival between *S. typhimurium* and *E. coli* were due to differences in diluting solution. Although efforts were made to use the same recipe of Ringer's solution as used by Meynell (1938), details of the solution were lacking in the paper. In addition, the method of chilling samples of *E. coli* used by Meynell involved cooling 25 % Ringer's solution next to the freezer compartment in a domestic refrigerator, which may have led to fluctuations or inaccuracies in the actual temperature. In the present study, the diluting solution for samples of *S. typhimurium* was chilled to 4°C in an electronically temperature controlled water bath, for at least 1 hour prior to adding bacterial cells, thus eliminating any temperature differences.

The role of σ^s in survival of *S. typhimurium* at 4°C was also examined in a strain (MPG480) that was deficient for RpoS. The overall pattern of survival at 4°C of a culture of MPG480 was similar to the wild type strain, SL1344. However, there was a marginal decrease in survival from 79 % for the samples from the SL1344 culture, to 64 % for the samples from the MPG480 culture, after incubation at 5 hours and subsequent chilling to 4°C. It was interesting that σ^s did not appear to play a major

role in survival of *S. typhimurium* at 4°C, and only had an effect during late exponential phase.

These studies bear some similarity to the examination of the influence of σ^S on culturability and viability of *S. typhimurium*, carried out by Munro & colleagues (1995), in which RpoS was found to play a major role in the culturability of *S. typhimurium* in high osmolarity seawater, in both exponentially growing and stationary phase cells. However, the RpoS effect in low osmolarity seawater was only observed if the culture was in stationary phase, which is in line with the findings for *S. typhimurium* in the present study. Furthermore, Munro & colleagues shifted the cultures of *S. typhimurium* from rich media at 37°C to seawater at room temperature. This may also have led to a temperature-dependent response, since RpoS production has been observed to increase, in a DsrA RNA-dependent manner, following a shift of an exponentially growing culture of *E. coli* from 30°C to 20°C (Sledjeski *et al.*, 1996). However, these experiments were carried out at 20°C, which is substantially higher than the minimal growth temperature of *E. coli* (approximately 8°C).Taken together, these data indicate that survival of *S. typhimurium* at 4°C involved RpoS, when the culture was entering stationary phase and that RpoS did not play a role in survival during exponential phase.

It is possible that proteins that are induced following temperature reductions are responsible for the diminished effect of σ^S. For example, the small, DNA binding protein, H-NS, is induced in exponentially growing *E. coli* cells, following a shift from 37°C to 10°C (La Teana *et al.*, 1991). Previous work on *E. coli* H-NS has shown that this protein binds directly to *rpoS* mRNA which results in an inhibition of translation. Analysis of an *E. coli hns* mutant which carries an insertional inactivation of the *hns* coding region, (*hns*::*neo*) indicated that σ^S accumulated to 10 fold higher levels in exponential phase, when the cells were grown at 37°C. However, the level then decreased to approximately the same as for the wild type cells in stationary phase. This points to a negative regulatory effect of H-NS on σ^S in exponential growth phase. Furthermore, this phenomenon appears to occur independently of the

growth rate or amino acid availability in minimal medium (Yamashino *et al.*, 1995). Thus, it is possible that increased expression of H-NS following a temperature decrease leads to a repression in the expression of σ^S.

Other factors that occur when the temperature is close to, or below, the minimum growth temperature may also affect σ^S expression. Changes in temperature have been correlated with proportional changes in the levels of the alarmone (p)ppGpp in *E. coli*, so that when the temperature decreases, the level of (p)ppGpp also decreases (Jones *et al.*, 1992). The alarmones are perhaps best known for their role during the stringent response, when levels of (p)ppGpp rise, in parallel with a co-ordinated decrease in the rate of synthesis of RNA and ribosomal proteins (Pao & Dyess, 1981). Interestingly, a positive correlation between ppGpp and σ^S levels has been reported. An *E. coli relAspoT* ((p)ppGpp-free) mutant exhibits a pleiotrophic phenotype similar to *rpoS* mutants in terms of the reduced σ^S level and the consequential effects. In addition, artificial overproduction of ppGpp results in an increase in the level of σ^S (Lange *et al.*, 1995). Gentry *et al.* (1993) also investigated whether *rpoS* is under positive stringent control and found that the expression of both transcriptional and translational *rpoS::lacZ* gene fusions was reduced in ppGpp free mutants. They also found that the *relAspoT* double mutation does not specifically influence transcription initiation at any of the 3 promoters upstream of *rpoS*, but rather affects elongation or transcript stability. Thus, reduction in the (p)ppGpp levels at low temperatures may also results in reduced expression of σ^S.

It has been suggested that the cold shock response is similar to a nutrient upshift response in terms of the levels of a decrease in the (p)ppGpp basal level together with increased synthesis of translational components (Jones *et al.*, 1992). A nutrient upshift results in an initial lag period before growth resumes. Although there is a lag before *de novo* protein synthesis is initiated, some protein synthesis does occur, which is most likely to be translation of long-lived mRNAs that were previously present in the cell. Thus, the stalling of translation occurs for newly synthesised mRNA transcripts (for a review see Kolter *et al.*, 1993). Shifting growing cultures of

E. coli from 37°C to 5°C, was shown to result in an accumulation of 70S ribosomal subunits and a block in translation initiation (Broeze *et al.*, 1978). It is interesting, then, that synthesis of some cold shock proteins has been shown to proceed in the presence of inhibitors of protein synthesis (0.1 mg ml^{-1} of kanamycin or 0.2 mg ml^{-1} of chloramphenicol). These inhibitors did not adversely affect either transcription or translation of *E. coli* CspA, CspB or CspG, when exponentially growing cultures were shifted from 37°C to 15°C (Etchegaray & Inouye, 1999). Thus, presumably, the specific proteins that are induced during the adaptation period have some means of overcoming the general block in translation initiation. This data supports the argument that the cold shock response is similar to a nutrient upshift.

Previous work on bacteria such as *E. coli* and *B. subtilis* has shown that exposure of the bacteria to a non-lethal stress can provide subsequent protection against an otherwise lethal level of the same stress. Furthermore, similar pre-adaptation to a non-lethal level of one stress can, in some cases, provide protection against exposure to another, otherwise lethal stress. Thus, the degree of protection afforded by adding 0.3 M sucrose to chilled Ringer's solution was examined in *S. typhimurium* SL1344. In addition to monitoring survival samples diluted into Ringer's solution, survival was also monitored from samples diluted into 25 % Ringer's containing 0.3 M sucrose. The addition of sucrose provided some degree of protection against the decrease in survival of the samples taken from the culture in early exponential phase (table 5.2 and figure 5.2). In fact, survival from the samples taken at 90 minutes was 63 % in the presence of sucrose compared to 6 % in the absence of sucrose. In contrast, Meynell's studies with *E. coli* showed that that addition of 0.3 M sucrose conferred complete protection against a much more substantial loss of colony forming ability, regardless of growth phase. It is possible that the differences in degree of survival are due to variations in the diluting solution or in the method of chilling. It should also be noted that the assay may not solely reflect the action of rapid chilling of colony forming ability in either the present study or those of Meynell. There was a small difference in osmotic pressure between NB medium (which was used for growth) and 25 % Ringer's solution (which was used as a

diluent). The solutions contained 5 g l^{-1} NaCl and 2.5 g l^{-1} NaCl, respectively. Thus, in addition to experiencing a sudden cold shock, the samples also experienced an osmotic down-shift. However, since unchilled samples were also diluted into 25 % Ringer's solution, the osmotic effect was accounted for, although the difference may have affected the results of the protection experiment. It should not be overlooked that dilution into Ringer's solution may also have elicited a starvation response. If this were the case, then the bacteria would have experienced both stresses at the same time. Thus, both a cold shock response and a starvation stress response would have been initiated. A key feature of the starvation response is the expression of the general stress sigma factor, σ^S (Lange & Hengge-Aronis, 1991). However, these studies have shown that σ^S does not appear to play a role in survival of *S. typhimurium* following a sudden downshift in temperature. Furthermore, separate studies have shown that σ^S did not play a major role in the regulation of *S. typhimurium cspB* at low temperatures. Thus, it would seem that the starvation response and the cold shock response have distinct forms of regulation.

Several studies have shown that *Salmonella* subspecies can be induced into a reversible VBNC state, and that exposure to low temperature is an important mechanism for induction. Furthermore, the time of induction of the VBNC of *Salmonella* seems to decrease as the ambient temperature decreases (Chmielewski & Frank, 1995; Smith *et al.*, 1994). These studies indicate that induction may have occurred during the rapid chilling assay. However, since these assays involved a relatively short, low temperature incubation time, it would be expected that the majority of the population remained culturable. In order to address this question more fully, it would have been necessary to take direct viable counts. Not only would this information resolve the question of culturability, it would also highlight any potential differences in culturability that had arisen from differences in growth phase.

When *E. coli* cells are osmotically challenged, the response involves uptake of compatible solutes (either by transport or *de novo* synthesis) that maintain the osmotic pressure of the cell. At least 20 genes are known to be subject to osmotic

control in *E. coli* (Hengge-Aronis, 1996). A well characterised example is the transport system for glycine betaine and proline, encoded by the *proU* operon (Baron *et al.*, 1987). It has been proposed by Jones *et al.*, 1992, that a sudden decrease in temperature induces a physiological state where the translational capacity of the cell is insufficient relative to the supply of charged tRNAs. Thus, it follows that translation of genes that are not cold inducible is delayed following cold shock. It is possible, therefore, that *S. typhimurium* osmotic stress responsive operons are not expressed efficiently following a sudden downshift in temperature, when the cells are growing exponentially. However, there is evidence to show that glycine betaine is actively transported at low temperatures in *Listeria monocytogens* (Ko *et al.*, 1994). These studies showed that glycine betaine is accumulated at approximately 5-fold higher concentration at 4°C relative to the level detected at 30°C. Furthermore, addition of 8 % NaCl, which stimulates transport of glycine betaine 20-fold at 30°C, resulted in a further 6-fold increase in the accumulation of this osmolyte at 4°C. Recent studies have shown that a *Listeria monocytogenes* transport system for glycine betaine (which is osmotically activated but does not require a high concentration of sodium ions for activity) shares a high degree of homology with *E. coli proU* (Ko & Smith, 1999). *S. typhimurium* possesses a similar *proU* driven glycine betaine transport system, therefore, there remains that possibility that glycine betaine is similarly accumulated in *S. typhimurium* at low temperatures (for a review, see Csonka & Hanson, 1991). This possibility, and whether glycine betaine confers a similar level of cryotolerance as that observed for *L. monocytogenes*, has yet to be determined.

In summary, survival of *S. typhimurium* at 4°C was, in part, growth phase-dependent, so that cells in stationary phase and lag phase were completely resistant to the cold shock. Furthermore, addition of an osmoprotectant, 0.3 M sucrose, led to partial protection for cells in exponential phase. Both sets of data, from *S. typhimurium* and *E. coli* (Meynell, 1938), support the large body of work on increased resistance of bacteria in stationary phase, to a variety of environmental stresses. In order to gain further insight into survival during cold shock, the effects of different diluents and

the roles of different cold shock inducible genes should be explored. In addition, the VBNC effect should be taken into account, so that viability is determined in addition to culturability. The method developed by Kogure *et al.* (1978) in which viability is determined by measuring cell elongation in the presence of naladixic acid, has been used previously for *S. typhimurium* (Smith *et al.*, 1994).

CHAPTER 6

CHANGES IN PROTEIN EXPRESSION DURING THE COLD SHOCK RESPONSE OF *S. typhimurium*: PROTEOMIC ANALYSIS

6.1 INTRODUCTION

6.1.1 Bacterial stress responses.

A key feature of bacteria is their ability to adapt to environmental challenges, by eliciting a stress response. Environmental changes that are known to induce stress responses include exposure to high and low temperature, low nutrient levels, UV irradiation, changes in osmolarity or pH, or high levels of oxidation. Such stress responses are commonly referenced against the so-called 'normal' situation of laboratory conditions, in other words under conditions that elicit healthy growth rates, i.e. in nutrient-rich broth, at 37°C with aeration. However, it is of note that bacteria are often found in environments that are classed as stressful and may require persistence under such conditions for long periods of time. Thus it is important to appreciate that all stress responses are relative and should probably be termed adaptation responses. None-the-less, characterisation of stress responses is fundamental to understanding how pathogens such as *Salmonella* adapt to and survive in a range of very different environments. Such adaptation has direct implication on the success of these pathogens.

A major feature of a stress response is a change in protein synthesis. Initially, the synthesis of some proteins decreases, while co-incident synthesis of specific stress-induced proteins increases. Frequently these stress-induced proteins play key roles in the stress response. Following this stage of acclimation, for the majority pre-stress protein synthesis resumes to a pre-stress level, providing that adaptation has been successful. However, if the bacteria cannot adapt or tolerate the new environment, the consequences are likely to become lethal and protein synthesis ceases. Bacteria that are in the stationary phase of growth normally exhibit a very low level of protein synthesis and can be considered to be in a semi-protected state (this chapter of this thesis).

An example of a well-characterised stress response is the heat shock response (Bakau, 1993). The heat shock response occurs following temperature upshift and is

particularly apparent when bacteria experience temperatures which exceed the optimum for growth. For example, shifting cultures of *E. coli* to a temperature such as 42°C results in elevation of a set of heat shock proteins. A sigma factor, σ^{32} encoded by *rpoH*, is responsible for the induction of many of these stress proteins. Such proteins include the protein chaperones, GroEL and DnaK, that aid protein folding at high temperatures (Bakau, 1993; Neidhardt & VanBogelen, 1987). Other stress responses that have been well studied include responses to changes in osmolarity (Hengge-Aronis, 1996), pH (Lee *et al.*, 1995), oxidation (Jenkins *et al.*, 1988) and carbon starvation (Spector & Cubitt 1992; Jenkins *et al.*, 1990).

As in studies of the heat shock response, several key proteins have been identified that are involved in adaptation to the change in conditions. In all of the aforementioned responses, the alternative, or general stress sigma factor, σ^{s}, has been shown to play a regulatory role. Some proteins have also been identified that are induced by several responses. These include some of the heat shock proteins and UspA, the universal stress protein. UspA was shown to be induced between 3 and 9-fold approximately 20 minutes after exposure to stresses, such as carbon starvations, oxidative shock or osmotic shock (Nystrom & Neidhardt, 1992). Interestingly, UspA was not induced following a downshift in temperature from 37°C to 10°C (Nystrom & Neidhardt, 1992). Induction of *E. coli uspA* was also examined in the presence of various toxic compounds, using a bioluminescent reporter system (Van Dyk *et al.*, 1995). Exposure to any one of a large number of toxic compounds (including propanol, phenol, copper sulphate and mercury) induced bioluminescence from the *uspA*::*lux* fusion.

Studies by Spector and colleagues (1986) examined the effects of phosphate, nicotinate, glucose or nitrogen starvation, and anaerobic or heat shock in *S. typhimurium*. They found that while there was a sizeable overlap between the proteins induced under the various starvation conditions, there was a less of an overlap between the starvation induced proteins and heat shock or anaerobic shock proteins. Only 9 proteins were detected that were induced by both starvation and either heat shock or anaerobic shock, whilst in contrast, 16 proteins were detected

that were common to more than one type of starvation (Spector *et al.*, 1986). Thus, there appear to be substantial differences in the regulation of components involved in different stress responses.

6.1.2 The effects of temperature on bacterial growth and survival.

Changes in temperature outside the optimal growth temperature range result in dramatic changes in bacterial growth rate (Stanier *et al.*, 1984). The effect of temperature on growth rates is described by the Arrhenius equation (Arrenhius, 1889):

$$r = A \exp^{Ae/RT}$$

where r is the bacterial growth rate, A is the Arrenhius constant, exp is the exponential, Ae is the activation energy, T is the absolute temperature and R is the universal gas constant. Over the temperature range of 20°C to 41°C, the logarithmic growth rate of *E. coli* is inversely proportional to the absolute temperature. However, outside this range, growth rate is less than predicted by the Arrhenius relationship.

In addition to changes in growth rate, alterations in temperature also result in many physiological parameters, such as ribosomal, functional, transcriptional and cell division (for a recent review, see Panoff *et al.*, 1998). Changes in lipid composition and membrane fluidity are also of key importance and a temperature downshift leads to a reduction in membrane fluidity. This has been well studied in cyanobacteria such as *Anabaena variabilis* and *Synechocystis* PCC 6803 where such membrane changes are rectified by desaturation of fatty acids which increases the proportion of *cis*-unsaturated fatty-acyl groups in the membrane lipids. (Sato & Murata, 1981; Wada & Murata, 1990). Moreover, in the case of *Synechocystis*, the mRNA transcript of *desA*, which encodes fatty acid desaturase, was shown to increase dramatically following a shift from 37°C to 22°C and when the ambient temperature increased again, membrane fluidity has been shown to increase (Vigh *et al.*, 1998).

6.1.3 Changes in protein expression following a decrease in temperature.

The cold shock response is elicited in both Gram negative and Gram positive bacteria following a downshift in temperature. This response is a typical stress response in that the changes in physiology and gene expression that occur aid in adaptation to persistence at low temperatures (for a recent reviews see Panoff *et al.*, 1998 and Yamanaka *et al.*, 1998). The response involves dramatic changes in the cellular composition of proteins of bacteria. While the majority of *de novo* protein synthesis decreases, transient induction of a set of proteins occurs, termed cold-induced proteins (CIPs) (Jones *et al.*, 1987). Within this group, small, hydrophobic, cold shock proteins (CSPs), such as CspA, are very highly induced (Goldstein *et al.* 1990). In *E. coli*, an initial lag in growth occurs following a decrease from 37°C to 15°C or 10°C, during which time CIPs are induced. However, some authors have termed this period as an acclimation phase, and this may result in some confusion. A second set of proteins, sometimes termed cold acclimation proteins (CAPs), is synthesised continuously during prolonged incubation at sub-optimal temperatures (Panoff *et al.*, 1997). For the purposes of this thesis, the terminology adopted by Panoff and colleagues has been adopted. Thus CIPS are the transiently induced proteins which include members of the CspA family and CAPS are continuously synthesised during incubation at sub-optimal temperatures.

Jones and colleagues (1987) reported dramatic changes in the synthesis of individual proteins following a shift in temperature of an exponentially growing culture of *E. coli* from 37°C to 10°C, using 2-D PAGE. They found that following the temperature reduction, the majority of proteins that were evident at 37°C were repressed. Indeed, only 28 proteins were detected after 2 hours and of this set, synthesis of 5 was reduced by the cold shock. The differential synthesis of 10 remained the same as that at 37°C and 13 were induced by the cold shock. CspA, identified by N-terminal sequencing in a later study, was the most highly induced cold shock protein. The relative rate of synthesis of 12 of the 13 cold shock proteins increased between 2- and 10- fold, whereas the level of CspA increased approximately 200-fold. Several

of the proteins were identified by co-migration of purified protein on 2-D gels, shown in table 6.1.

A separate study has shown that a temperature downshift from 37°C to 13.5°C resulted in a decrease in overall protein synthesis of exponentially growing *E. coli* cells. This was co-incident with an increase in synthesis, of 2-fold or more, of 22 proteins (Herendeen *et al.*, 1979) (these proteins are also included in table 6.1). The unknown proteins that were also cold shock inducible are: B018.4, C056.0, D044.5, D074.0, D099.0, F023.3, F038.0, F082.5, F178.0, G027.2, G029.6, G041.2 (the proteins are numbered according to the alpha-numeric spot naming system used in VanBogelen, *et al.*, 1996).

6.1.4 Changes in expression of CspA-like proteins following a decrease in temperature.

For a number of different bacteria, genetic analysis and analysis of protein profiles by 2-D PAGE following a temperature reduction, have provided evidence for an increase in cold shock-inducible proteins, including synthesis of members of the CspA family (for a recent review see Yamanaka *et al.*, 1998). Moreover, to date, *cspA*-like genes seem only to be lacking from only a small number of bacteria whose genomes have been sequenced, including *Helicobacter pylori* and *Mycoplasma genatalium* (Tomb *et al.*, 1997, Fraser *et al.*, 1995). To maintain consistent terminology, the major CSP has been termed CspA, except in the case of *B. subtilis*, where the major CSP has been termed CspB (Graumann *et al.*, 1994).

Protein	Function	*S. typhimurium* equivalent [a]
RecA[1]	DNA recombination and repair	93%
GyrA[1]	DNA supercoiling	73 %
H-NS[1]	Involved in chromosome organisation of the nucleoid.	91 %
Pnp[1]	Degradation of mRNA	96 %
NusA[1]	Termination of transcription	90 %
IF-2	Binding of charged tRNA-f-met to 30S ribosomal subunit	86 %
CsdA[3]	ATP-dependent RNA helicase, DEAD-box, (previously designated DeaD)	96 %
RplL[2]	50S ribosomal subunit protein L7/L12	
RbfA[4]	Involved in ribosomal maturation or initiation of translation	97 %
Trigger Factor[5]	Chaperone associated with GroEL, prolyl-isomerase activity	78 %
IbpB[2]	HSP20 homologoue	50 %
Hsc66[6]	DnaK homologoue	82 %
OmpA[2]	Outer membrane protein	91 %
SodA[2]	Superoxide dismutase, manganese	81 %
CarA[2]	Carbamoyl-phosphate synthetase	94 %
MetE[2]	Tetrahydropteroyltrigluta-mate methyltransferase	83 %
ArgI[2]	Ornithine carbamoyltransferase I	89 %
AceE[1]	Decarboxylation of pyruvate	78 %
AceF[1]	Dihydrolipoamide acetyltransferase subunit of pyruvate dehydrogenase	72 %
SdhA[2]	Succinate dehydrogenase, flavoprotein subunit	72 %
SucA[2]	2-oxoglutarate dehydrogenase (decarboxylase component)	87 %
SucB[2]	2-oxoglutarate dehydrogenase (dihydrolipoyltrans-succinase E2 component)	83 %
CspA[7]	major cold shock protein	100 %
CspB[8]	CspA homologoue	
CspG[9]	CspA homologoue	unknown
CspI[10]	CspA homologoue	unknown

Table 6.1 Identified *E. coli* proteins whose synthesis increases following incubation at 10°C or 15°C. (1, Jones *et al.*, 1987; 2, Herendeen *et al.*, 1979; 3, Jones *et al.*, 1996; 4, Jones & Inouye, 1996; 5, Kandor & Goldberg, 1997; 6, Lelivelt & Kawula, 1995; 7, Goldestein *et al* 1990; 8, Lee *et al.*, 1994; 9, Nakashima *et al.*, 1996; 10, Wang *et al.*, 1999). a – The percentage relationship (identity) between members of the *S. typhimurium* and *E. coli* CspA family is based on the *S. typhimurium* sequences in the Washington University School of Medicine database.

In a recent study by Etchegaray and colleagues (1996), 2-D PAGE was used to investigate differential cold shock induction of CspA and CspB in *E. coli*. Exponentially growing cultures were inicubated with ^{35}S-methionine following temperature reductions from 37°C to 30°C, 24°C, 20°C, 15°C, 10°C or 6°C, and the protein extracts were resolved on 2-D gels. They found that at 30°C, a low level of CspA was detected but CspB was not apparent. The production of CspA became prominent at 24°C and was maintained at a high level between 20°C and 10°C. In contrast, CspB production was observed at 20°C and peaked at 15°C. At 6°C, production of CspA and CspB was still evident although the level of each protein had decreased sharply. These results are summarised in table 6.2. Overall, the results indicated that the temperature dependence of CspA production was broad relative to CspB production.

Temperature (°C) after reduction from 37°C	*E. coli* CspA	*E. coli* CspB
6	0.05	0.05
10	0.7	0.3
15	1.0	1.0
20	0.9	0.4
25	0.8	0.1
30	0.2	0.1

Table 6.2 Differential cold shock induction of *E. coli* CspA and CspB.

The cold shock-inducible proteins were detected by 2D electrophoresis following temperature shifts from 37°C to those indicated. The level of protein was analysed by a phosphorimager. The level of induction is shown relative to the highest level (at 15°C). Adapted from Etchegaray *et al.*, (1996).

6.1.5 Changes in protein expression following entry in stationary phase.

The stationary phase sigma factor, σ^S, is known to regulate more than 30 genes on entry into stationary phase and the majority of these are positively regulated (for a review, see Loewen & Hengge-Aronis, 1994). These proteins are frequently known as Pex proteins (post-exponential phase) and some of them are list below (table 6.3).

Protein (encoded by)	Function	*S. typhimurium* equivalent [a]
HPII (*katE*)	catalase required for degradation of hydrogen peroxide.	81 %
Dps (*dps*)	DNA-binding protein involved in stationary phase oxidative stress response	95 %
PBP6 (*bolA*)	penicillin binding, involved in cell wall synthesis at the septum	93 %
SpvA (*spv*)	part of the virulence *spvABCD* operon	*
OtsA (*otsBA*)	involved in trehalose synthesis, used as an osmoprotectant.	80 %
OtsB		69 %
GlgS (*glgS*)	involved in glycogen synthesis	72 %
AppY (*appY*)	acid phosphatase regulator, anaerobically induced	41 %
OsmB (*osmB*)	outer membrane protein involved in stationary phase membrane alterations	95 %

Table 6.3 A selection of known *E. coli* σ^S controlled proteins and their associated functions. (For a review of σ^S regulated proteins see Loewen and Hengge-Aronis, 1994). a - The percentage relationship (identity) between *S. typhimurium* and *E. coli* proteins is based on the *S. typhimurium* sequences in the Washington University School of Medicine database. * - The *spv* operon is specific to *Salmonella* sub-species.

6.1.6 Proteomics.

The proteome is the term used to describe proteins expressed by the genome, but unlike the genome, the proteome is not a fixed feature of an organism. It changes with the state of development or environmental conditions. 2-Dimensional polyacrylamide gel electrophoresis (2-D PAGE) separates proteins initially on the

basis of their isoelectric point, followed by resolution by molecular size. This system can simultaneously resolve up to thousands of proteins and thus is a key approach to unravelling proteomes. Different combinations of pH gradient and polyacrylamide gel porosity enable the study of the vast majority of cellular proteins and when 2-D PAGE is used in conjugation with powerful micro-sequencing techniques, such as nanospray ionisation and tandem mass spectrometry (Schevchenko *et al.*, 1997), it provides an invaluable tool for identifying the nature of many of these proteins.

The potential of 2-D PAGE was realised in the early 1970s. Indeed, the methods currently used are essentially those as described by O'Farrell in 1975. At this time, authors such as O'Farrell recognised that thousands of proteins could be separated and potentially identified. However, without the present-day wealth of molecular biological knowledge, 2-D PAGE was mainly used for analysing patterns of protein expression and tools for sequencing low levels of proteins.

2-D PAGE is now used routinely to study changes in proteomes, following protein modification or exposure to environmental stresses. This may be achieved by radioactively labelling protein samples at time points before, during and after the stress and resolving the proteins by 2-D PAGE. The protein profiles or maps are then compared to the protein profile of the reference gel and changes in protein expression are identified. Technical improvements have led to good reproducibility between 2-D gels, facilitating comparison with standard reference gels. For example, pre-cast iso-electric focusing (IEF) strips with immobilised pH gradients are commercially available, which greatly reduces differences arising from variations inherent in pouring or running IEF gels (Hanash *et al.*, 1987). Pre-cast gels are also available for the second dimension, again reducing variability between gels. Such improvements have led to an increase in the production of 2-D reference maps. Some notable examples include; the investigation starvation, anaerobiosis and heat shock inducible proteins in *S. typhimurium*, described earlier (Spector *et al.*, 1986). Earlier studies identified transient induction of 30 *S. typhimurium* proteins following exposure to 60 μM H_2O_2, at 37°C. 5 of these proteins were also induced 10 minutes after the culture was shifted from 28°C to 42°C (Morgan *et al.*, 1986). The high quality of the 2-D

PAGE profiles has led to formation of protein reference maps. For example, the assignment of a reference map for cell envelope proteins in *S. typhimurium* (Qi *et al.*, 1996) and the *E. coli* gene-protein index (Neidhardt *et al.*, 1983).

Key proteins detected in this manner that are involved in stress responses, may be excised, either directly from the gel or following transfer to a solid support membrane, and sequenced. The choice of the sequencing method is largely determined by the abundance of the protein. N-terminal sequencing, for example, using automated Edman degradation, is used for abundant proteins (Wilkins *et al.*, 1998). However, this technique is expensive and required tens of nanograms of protein. An alternative is to sequence peptide fragments using extremely sensitive mass spectrometry techniques. In the latter use, peptide fragments are generated after gel-extraction, by enzymatic digestion of the protein, e.g. with trypsin, and very accurate peptide masses are determined using either MALDI TOF MS or NS/MS (matrix assisted laser desorption/ionisation time of flight mass spectrometry and nanospray ionisation mass spectrometry, respectively). Essentially, in both techniques, ions are generated from the peptide fragments and the ionic mass is measured in either a flight detector or a quadrupole. The main difference lies in sample delivery; the samples are in solid phase for MALDI TOF MS and in liquid phase for NS MS. In addition, NS MS can measure peptide masses from microlitre quantities within 1 hour (Schevchenko *et al.*, 1998). The peptide masses are finally matched against peptide mass databases, which may result in protein identification. If the concentration of the protein is particularly low, an alternative method of identification is the use of protein immunodetection with an appropriate antibody.

6.1.7 Aims of this chapter.

The majority of reports of protein synthesis during the cold shock response relate to exponentially growing bacteria, above their respective minimum growth temperatures. At the time of writing, there are no reported studies of protein synthesis during the cold shock response when cultures are in stationary phase. Yet, it is well known that bacteria in stationary phase have a far higher rate of survival

when encountering environmental stresses (Kolter *et al.*, 1993). This is especially relevant to the food industry, since bacteria are likely to be in different growth phases when associated with food products. In the absence of such information, the capacity for inducing a cold shock response was explored using stationary phase cells of the pathogen *S. typhimurium* from strain SL1344. In particular it was of interest to known whether there were any parallels in stress protein synthesis between exponentially growing cultures and stationary phase cultures.

Another important consideration for the food industry is the storage temperature, which is legally required for fresh meat and poultry. This is normally 4°C, and below the minimum growth temperature for *S. typhimurium* (of approximately 8°C). Thus, *de novo* protein synthesis by SL1344 was examined at 10°C and 4°C, above and below the minimum growth temperature. In addition, *de novo* protein synthesis of exponentially growing MPG361 (*cspB*::Mu*dlux*) cells was examined following a shift from 30°C to 10°C to determine the effects of the CspB mutation on CSP production. This strain contains a bioluminescent reporter system inserted adjacent to the 23rd codon of *S. typhimurium cspB* (Craig *et al.*, 1998). Light reporter studies have shown that *cspB* is expressed both in exponential and stationary phase, both at 10°C and 4°C (see chapter 4 of this thesis). However, the effect of a *cspB* mutation on expression of CspA has not yet been determined.

6.2 RESULTS

The molecular basis of the cold shock response of bacteria has only recently been investigated. In particular, studies have focused on *E. coli* (Jones *et al.*, 1987) and *B. subtilis* (Willimsky *et al.*, 1992), although this response has been reported for many other types of bacteria. There are only a few reports of the cold shock response of *S. typhimurium*, and these have documented thermoregulation of *S. typhimurium cspB* (Craig *et al.*, 1989) and aspects of the cold shock response of *S. enteritidis* (Jeffreys *et al.*, 1989). *S. typhimurium* is an important food-borne pathogen which is able to survive on refrigerated food-stuffs, thus increasing the risk it poses. It is important that the cold shock response of *S. typhimurium* is fully characterised in order that strategies can be designed to minimise their persistence on foodstuffs.

This section of the thesis characterises *de novo* protein synthesis of *S. typhimurium* at low temperatures, using 2-D PAGE. Samples of the cultures were incubated with ^{35}S-methionine to label newly synthesised proteins prior to the temperature downshift and after 1.5 and 4 hours at 10°C or after 2, 6, 12, 24 and 96 hours at 4°C. A selection of incubation times was examined in relation to specific temperatures or growth phases, so that samples in exponential phase were incubated with ^{35}S-methionine for 5 minutes at 37°C, or 30 minutes at 10°C or 4°C. Samples that were in stationary phase were labelled with ^{35}S-methionine for 10 minutes at 37°C, or 60 minutes at 10°C or 4°C. (This procedure, and those that follow, have been described in full in chapter 2 of this thesis).

Once the proteins were radioactively labelled, the cells were harvested and fractionated by sonication. Nucleic acids present in the samples were degraded with the appropriate enzymes, and the proteins from the whole cell extract were suspended in a 2-D PAGE buffer. Initially, proteins were separated on the basis of their isoelectric point, using pre-cast IEF strips with an immobilised linear pH gradient (pH 3 to 10). Subsequently, the proteins were separated on the basis of their molecular weight using SDS PAGE. A tricine buffered system was used for the 2nd dimension slab gels to resolve small molecular weight proteins, as described in Schagger and Von Jagow (1987). Following electrophoresis, the gels were dried and

autoradiographed. As the autoradiographic exposure time was the same for all the gels from each experiment, differential protein synthesis could be quantified. However, it should be noted that the labelling time at 37°C was only 16% that of the cold shocked samples, therefore the radioactive signal was a 6-fold underdisplay relative to the gels of the cold shocked samples. The autoradiographs were scanned into a computer and protein synthesis from the 2-D PAGE gels was analysed using Phoretix 2-D software. The following parameters were used for quantitative analysis of spot abundance:

- The radioactive signal derived from individual proteins was detected and analysed by computer analysis. The pixel volume of each protein was measured and given a 'protein spot volume'. This value was then used to determine the abundance of each protein. A protein spot was defined as having a minimum pixel size of 8.
- For each protein gel, the background was calculated as the 'lowest on boundary'. The smallest boundary intensity value for each spot was subtracted from every pixel making up the protein spot. Thus, the background value was 'local' for every protein spot in the autoradiograph.
- Once the spot volume was measured in each gel, the spots were matched to corresponding spots in the reference gel, and the relative levels of abundance were then calculated.
- A protein spot volume of 900 units was chosen as a threshold value for further analysis, since protein spots below this threshold were more difficult to measure and compare, and may therefore be prone to error during the analysis procedure.

6.2.1 Protein expression in exponentially growing SL1344 cells following a shift from 37°C to 10°C.

Exponentially growing cells do not have a high survival rate following exposure to sudden and severe environmental stresses. For example, exposing *S. typhimurium* to a sudden drop in temperature from 37°C to 4°C was found to result in a 96 % decrease in colony forming ability, when the cells were in exponential phase (see

chapter 5). In contrast, almost 100% of cells that were in stationary phase remained plateable. 2-D PAGE analysis has shown that key stress proteins are highly induced shortly after exposure to environmental stresses. Such proteins have, in some cases, been shown to play essential roles in stress adaptation (Graumann *et al.*, 1996).

Previous studies of the cold shock response of *E. coli* (for example; Jones *et al.*, 1987) and *B. subtilis* (for example; Graumann *et al.*, 1996) have focused on low temperatures where growth and division can still occur. Thus, once adaptation to the low temperature had occurred, the so-called 'house-keeping' proteins would be expressed in addition to the stress-induced proteins. However, since multiplication of *S. typhimurium* cannot proceed at 4°C (Mossel *et al.*, 1981), the effects of protein expression at this temperature were unknown. For this thesis, *de novo* protein expression of exponentially growing SL1344 was examined both at 10°C and 4°C, which are above and below the minimum temperature permissible for growth, respectively.

S. typhimurium SL1344 cells were grown in defined Spitzizen, at 37°C with shaking, to mid-exponential phase. When the $O.D._{600\ nm}$ reached 0.5, 3 ml aliquots of the culture were shifted to a water bath that was maintained at 37°C or 10°C. The cells were incubated with 24 μCi of $^{35}S^{met/cys}$ as described previously. Immediately following the period of radioactive labelling, the cells were disrupted by sonication and the soluble proteins from the whole cell extract were resolved by 2-D PAGE.

Shifting an exponentially growing culture of SL1344 from 37°C to 10°C or 4°C resulted in large changes in *de novo* protein synthesis. Table 6.4 details the numbers of radio-labelled proteins detected in autoradiographs from the different time points. Only proteins that were above the threshold level will be discussed further.

Conditions	Total number of proteins above threshold level
37°C 0 hours	180
10°C 1.5 hours	87
10°C 4 hours	188
4°C 2 hours	64
4°C 6 hours	21
4°C 12 hours	15
4°C 24 hours	25
4°C 96 hours	57

Table 6.4 The number of proteins that were detected from exponentially growing SL1344 cells at 37°C, 10°C and 4°C. The total number of proteins that lie above the threshold protein spot volume of 900 units is given.

The autoradiograph (figure 6.1) shows *de novo* proteins synthesis of an exponentially growing culture of SL1344 at 37°C. 180 proteins (above the threshold level) were detected over a pI range of 3 – 10 and between 2.5 KDa and 70 KDa. Shifting the culture of SL1344 from 37°C to 10°C resulted in a dramatic reduction in the level of protein synthesis, so that a total of 87 proteins above the threshold level were detected (the autoradiograph is shown in figure 6.2). 79 proteins were detected both at 37°C and after 1.5 hours at 10°C while 42 proteins were found to be repressed following the shift from 37°C to 10°C. The synthesis of several proteins increased following temperature the downshift. 14 proteins (8, 9, 10, 11, 13, 14, 15, 16, 17, 18, 19, 21, 22, 23) were induced at least 2-fold, from a low level at 37°C, and showed their highest induction in the sample after 1.5 hour at 10°C. An example is protein number 22, shown in figure 6.3, panel A. The synthesis of 6 proteins was shown to increase by more than 10-fold at 10°C. These proteins are shown on figure 6.2 and numbered as 1, 2, 3, 4, 5 and 6. Montages of proteins 1 and 5 are show in figure 6.3, panels B and C, respectively, as examples. Protein number 1 was induced approximately 200-fold and was found by N-terminal sequencing to encode CspA (discussed later in this chapter).

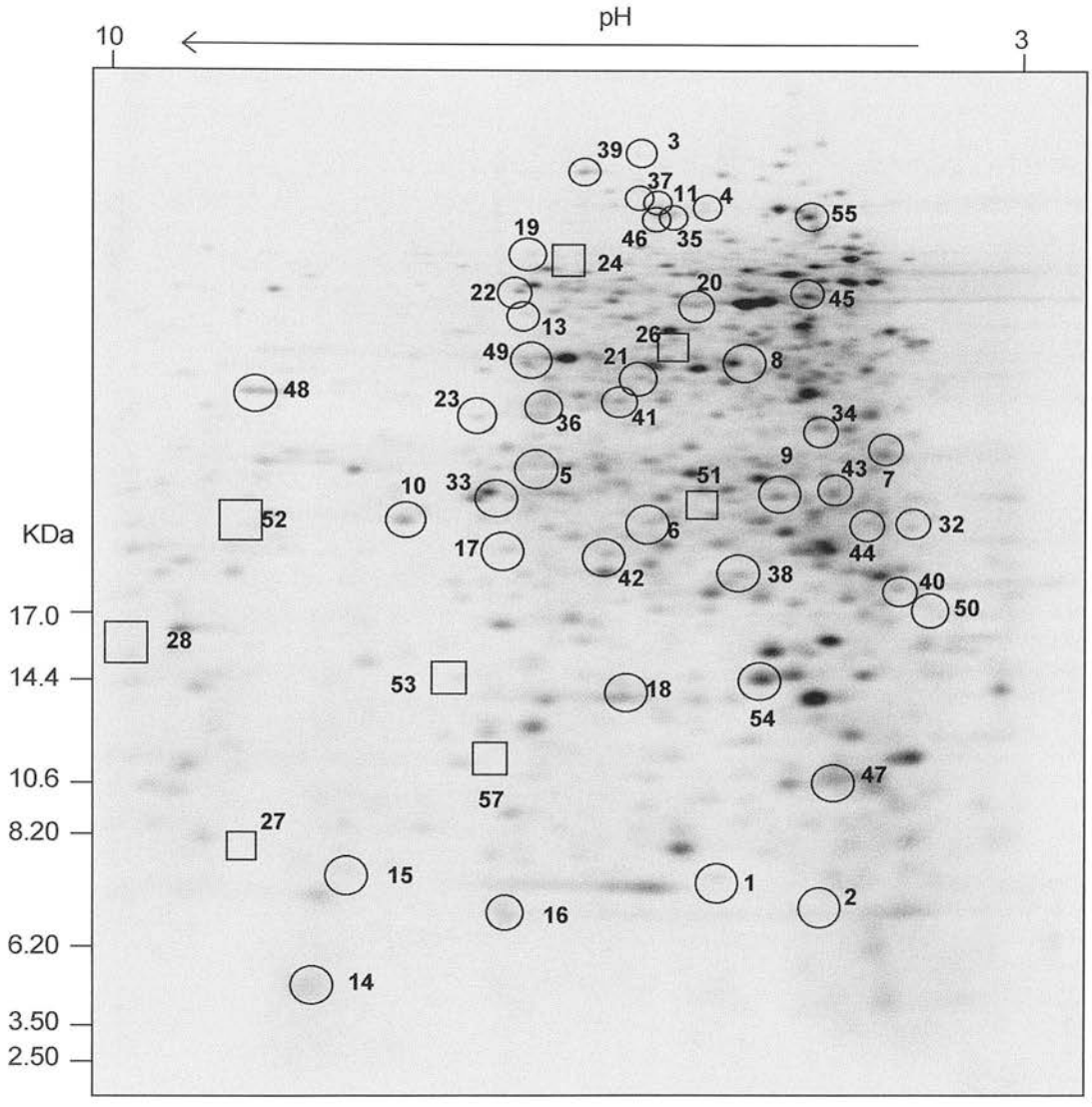


Figure 6.1 Autoradiograph of protein extracts from ^{35}S-methionine-labelled *S. typhimurium* SL1344 cells grown to mid-exponential phase at 37°C, in Spitizen medium. 3 ml of the culture was radioactively labeled for 5 minutes at 37°C. The cells were disrupted by sonication and the protein extracts were separated by 2-D PAGE. For the first dimension 110 mm pre-cast IPG strips, pH 3 to 10 were used for separation and for the second dimension 1 mm x 150 mm x 180 mm 16.5 % slab gels were used which resolved proteins between 2 and 70 KDa.

Protein spots whose syntheses were found to increase following a shift to low temperatures are circled. The positions of the boxes represent proteins that were only detected at low temperatures and were not evident at 37°C. The numbered proteins are listed in table 6.5.

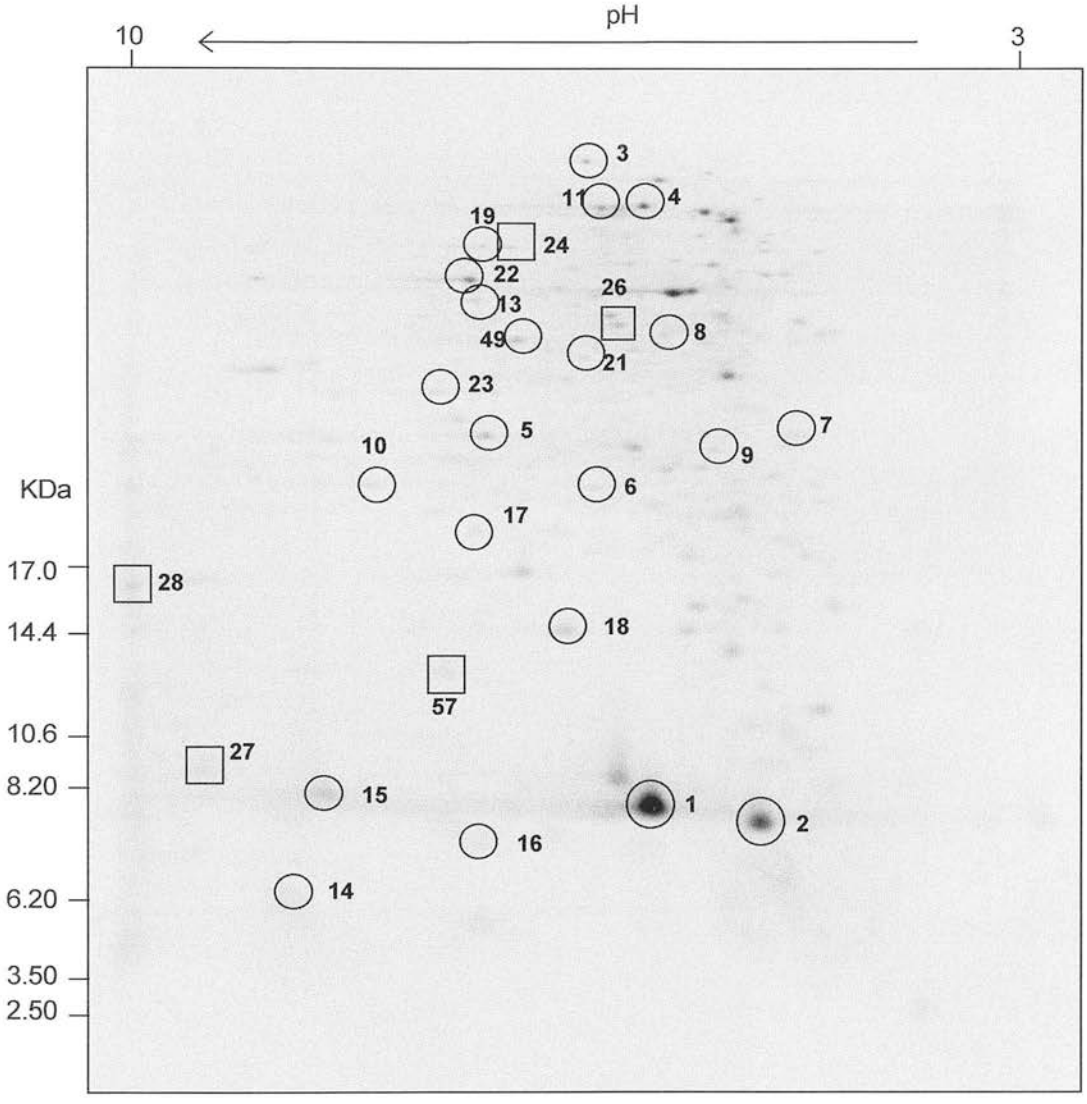


Figure 6.2 Autoradiograph of protein extracts from ^{35}S-methionine-labelled *S. typhimurium* SL1344 cells grown to mid-exponential phase at 37°C, in Spitizen medium, and incubated at 10°C for 1.5 hours. 3 ml of the culture was radio-actively labeled for 30 minutes at 10°C. Proteins from whole cell extracts were separated by 2-D PAGE as described in the legend for figure 6.1

Protein spots whose syntheses were found to increase following a shift to 10°C are circled. The positions of the boxes represent proteins that were only detected at 10°C and were not evident at 37°C. The numbered proteins are listed in table 6.5.

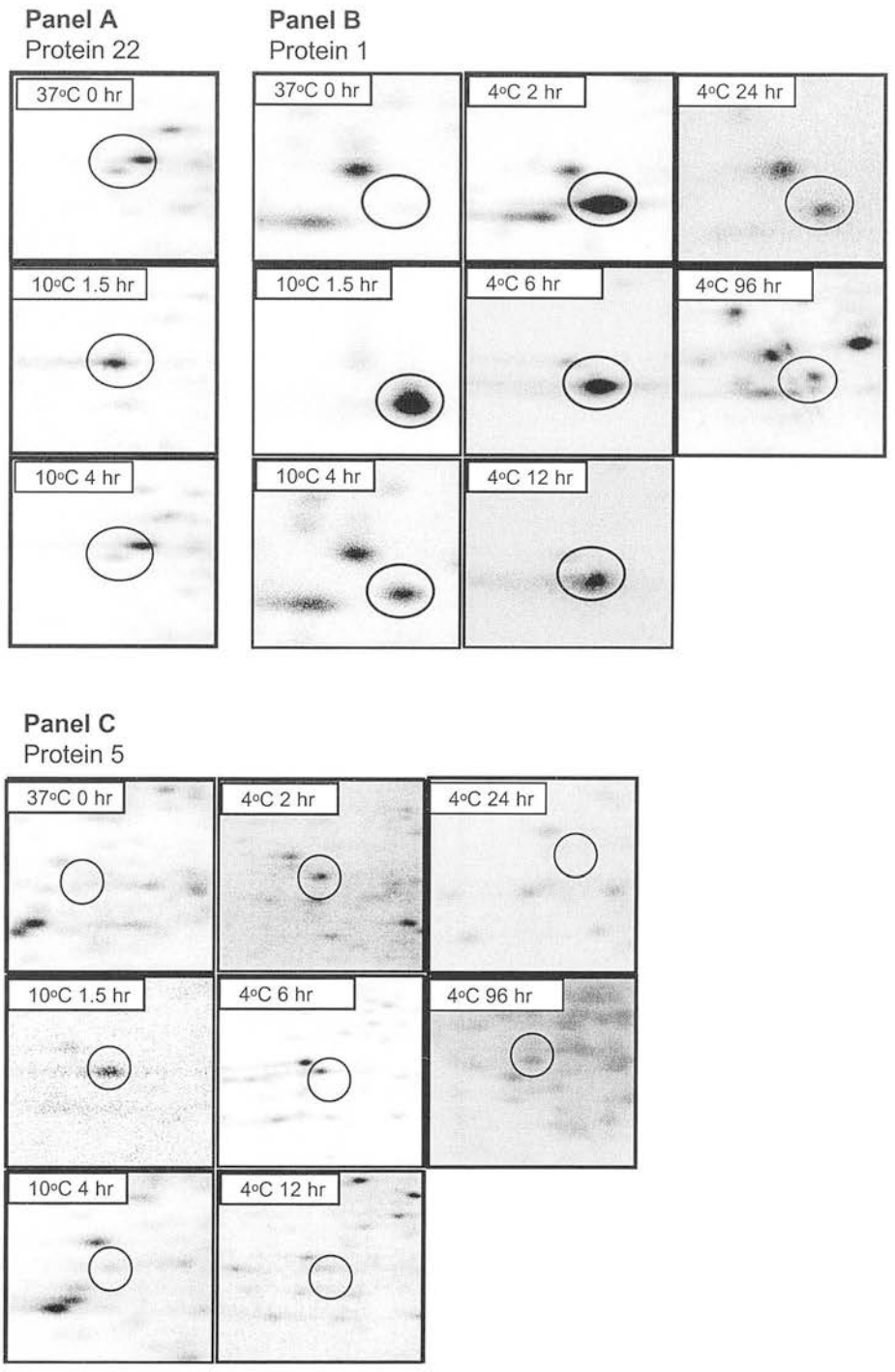


Figure 6.3

Montages showing cold induced proteins from exponentially growing SL1344 at 10°C and 4°C. Panel A: Protein 22 provides an examples of a CIP that was induced at least 2-fold. Panel B and C: Proteins 1 (CspA) and 5 (respectively) were induced more than 10-fold after incubation at 10°C for 1.5 hours.

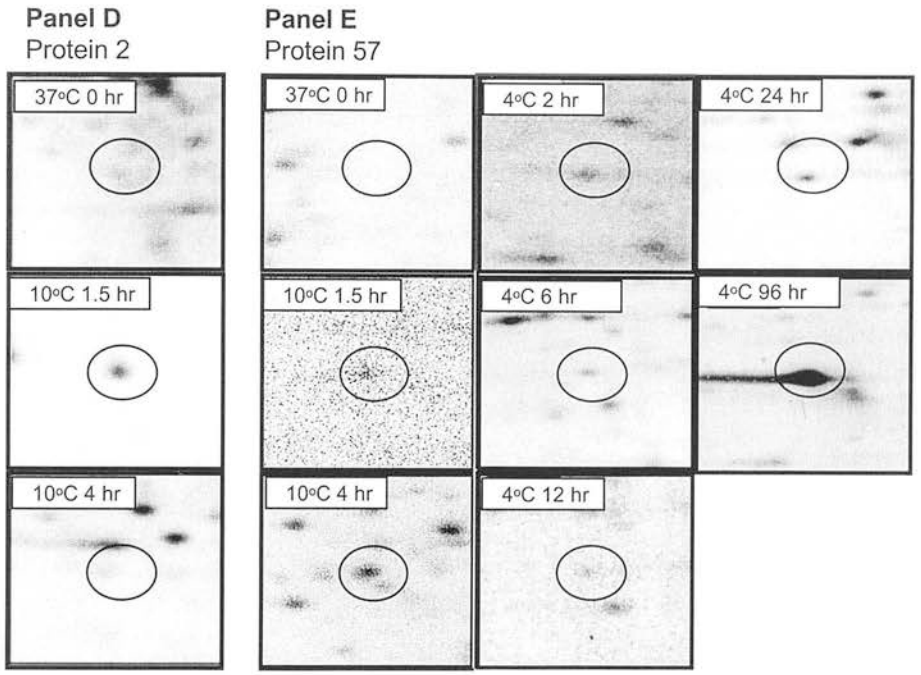


Figure 6.3 (continued)

Montages showing cold induced proteins from exponentially growing SL1344 at 10°C. Panel D and panel E: Proteins 2 and 57, respectively, were detected at 10°C, but were previously undetected at 37°C.

A set of 5 cold-induced proteins that were not detected at 37°C, but were detected at a high level after 1.5 hours at 10°C. These include proteins 24, 26, 27, 28 and 57 (figure 6.2). As an example, protein 57, is shown in figure 6.3, panel D. In addition, some proteins detected after 1.5 hours at 10°C, were absent after 4 hours at 10°C. An example is protein 2 shown in panel E of figure 6.3. Any remaining proteins, not accounted for, were below the threshold level of detection.

De novo protein synthesis was also examined after the exponentially growing culture of SL1344 was incubated at 10°C for 4 hours (the autoradiograph is shown in figure 6.4). In general, the protein map appeared similar to that at 37°C, prior to the temperature downshift. In fact, 188 proteins (above the threshold level) were detected after 4 hours at 10°C, which is similar to the level at 37°C.

The vast majority of the proteins at this time point matched those detected at 37°C, although 18 of these proteins (numbers 9, 19, 20, 23, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49) were induced between 2 and 10-fold, and 10 proteins (numbers 1, 3, 6, 10, 11, 21, 32, 33, 34, 35) were induced more than 10-fold, relative to the level detected at 37°C. The latter set of proteins included CspA (protein number 1). 102 of the proteins that were detected after 4 hours at 10°C were also detected after 1.5 hours at 10°C. Of these 40 proteins were induced more than 2-fold and the synthesis of 4 proteins (numbers 32, 35, 46) was induced more than 10-fold, relative to the level of synthesis detected after 1.5 hours at 10°C. The level of induction of proteins 21 and 10 increased by 27-fold and 33-fold, respectively, in relation to the level detected at 37°C (shown in figure 6.5, panels A and B, respectively). The level of synthesis of 12 proteins were found to have decreased at least 2-fold, relative to the level detected after 1.5 hours at 10°C. At this time point, CspA was repressed relative to the level after 1.5 hours at 10°C, however, synthesis was still 19-fold more that that at 37°C, (panel B in figure 6.3).

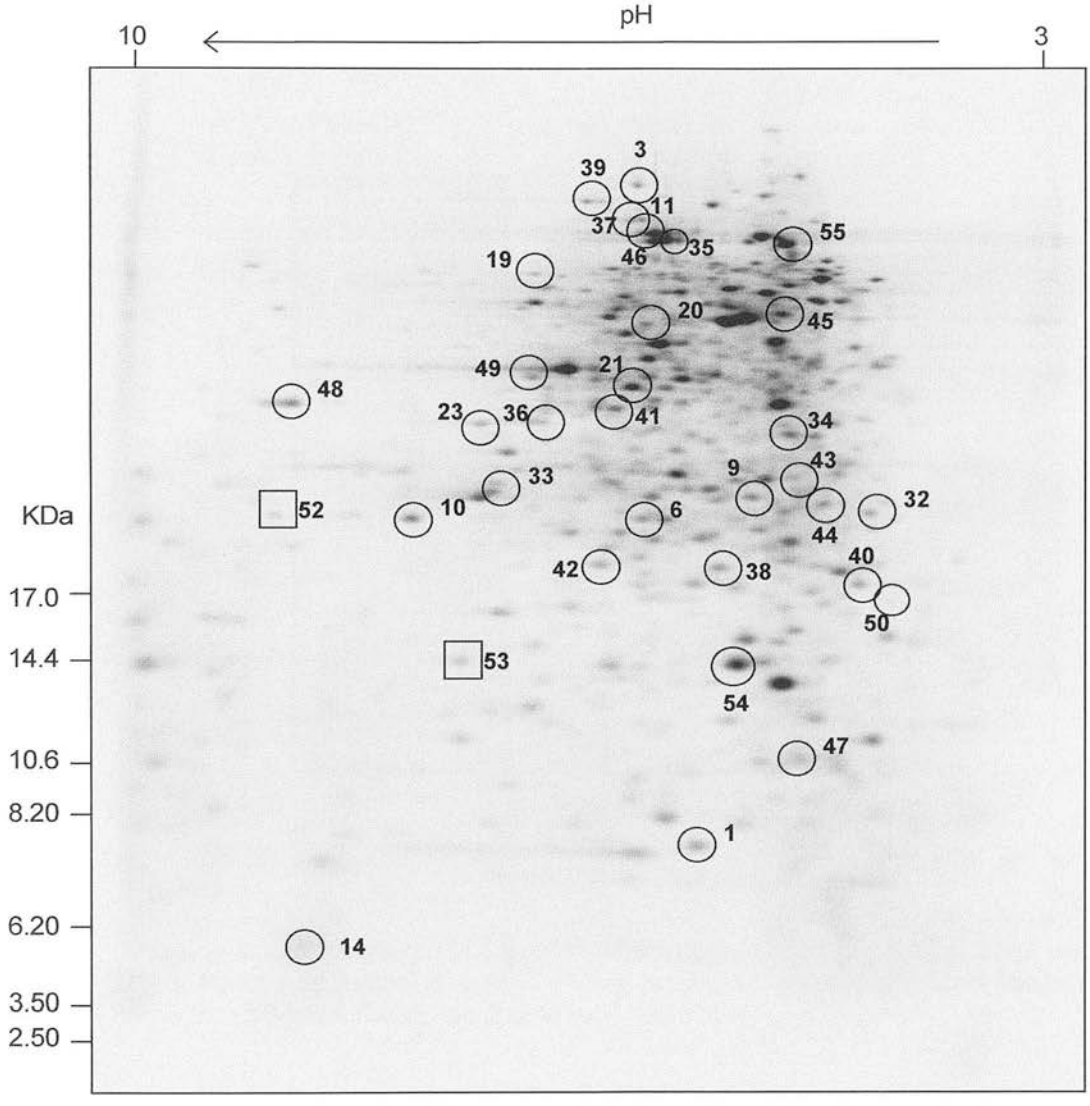


Figure 6.4 Autoradiograph of protein extracts from ^{35}S-methionine-labelled *S. typhimurium* SL1344 cells grown to mid-exponential phase at 37°C, in Spitizen medium, and incubated at 10°C for 4 hours. 3 ml of the culture was radio-actively labeled for 30 minutes at 10°C. The cells were disrupted by sonication and the protein extracts separated by 2-D PAGE. Proteins from whole cell extracts were separated by 2-D PAGE as described in the legend for figure 6.1

Protein spots whose syntheses were found to increase following a shift to 10°C are circled. The positions of the boxes represent proteins that were only detected at 10°C and were not evident at 37°C. The numbered proteins are listed in table 6.5.

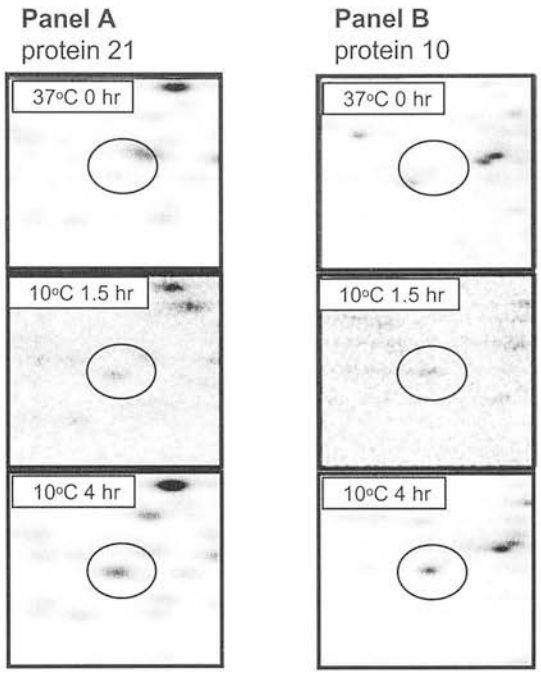


Figure 6.5

Montage showing cold shock induction of cold acclimation proteins at 37°C and 10°C. Panel A and panel B: protein 21 and protein 10, respectively, were induced more than 10-fold following incubation for 4 hours at 10°C.

6.2.2 Protein expression in exponentially growing SL1344 cells following a shift from 37°C to 4°C.

Exponentially growing cultures of SL1344 were also examined after incubation at 4°C to determine the complement of proteins that were induced below the minimum temperature permissible for growth. The cultures were grown to mid-exponential phase, as described previously. 3 ml aliquots were incubated at 4°C for 2, 6, 12, 24 or 96 hours and labelled with $^{35}S^{met/cys}$ for 30 minutes. Soluble proteins were extracted from the cells and resolved by 2-D PAGE as described previously.

Incubation for 2 hours at 4°C resulted in a decrease in overall *de novo* protein synthesis (figure 6.6) relative to that at 37°C. Indeed, only 64 proteins above the threshold level were detected. Relative to their level of abundance at 37°C, 4 proteins (5, 9, 15, 18) were induced between 2 and 10-fold after 2 hours at 4°C, while 3 proteins (1, 2, 34) were induced more than 10-fold (figure 6.6). The latter set of proteins included CspA, which was induced 134-fold relative to the level at 37°C, and was the most abundant protein detected at this time point. Protein 5 was induced more than 5-fold relative to 37°C and can be seen in figure 6.3 C.

40 proteins detected after incubation for 2 hours at 4°C were also detected after 1.5 hours at 10°C, although only protein 34 was found to have been induced relative to the level of synthesis after 1.5 hours at 10°C. As many as 27 proteins were repressed after incubation at 4°C, relative to the level detected after 1.5 hours at 10°C.

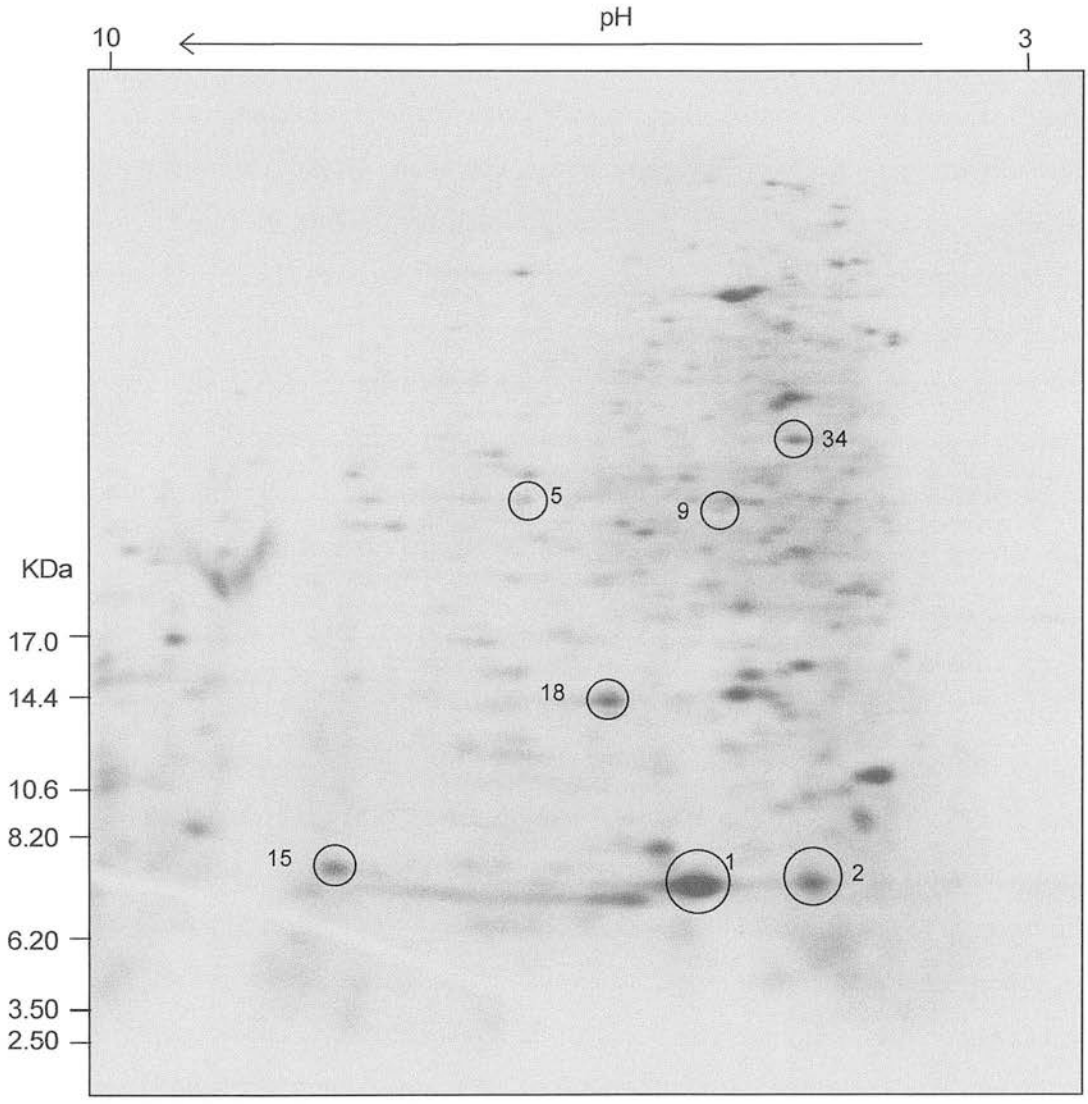


Figure 6.6 Autoradiograph of protein extracts from ^{35}S-methionine-labelled *S. typhimurium* SL1344 cells grown to mid-exponential phase at 37°C, in Spitizen medium, and incubated at 4°C for 2 hours. 3 ml of the culture was radio-actively labeled for 30 minutes at 4°C. The cells were disrupted by sonication and the protein extracts separated by 2-D PAGE as described in the legend for figure 6.1.

Protein spots whose synthesis was found to increase following a shift to 4°C are circled. These numbered proteins are listed in table 6.5.

Figure 6.7 shows *de novo* protein synthesis following incubation at 4°C for 6 hours. Overall protein synthesis was repressed relative to incubation for 2 hours at 4°C, so that only 21 proteins were detected above the threshold volume after 6 hours. CspA was still the most highly induced protein relative to 37°C, its level was approximately 90-fold higher than observed at 37°C. The relative level of synthesis of 3 proteins (5, 19, 33) increased between 2 and 10-fold at this point, compared with their level at 37°C. Protein 5 is shown as an example (panel C figure 6.3) and was induced more than 8-fold, relative to its level at 37°C. Compared to the level of protein synthesis detected after 2 hours at 4°C, protein19 was induced more than 10-fold, following incubation of 6 hours at 4°C. In addition, the level of synthesis of 5 proteins was found to have decreased relative to the level detected after 2 hours of incubation at 4°C.

Incubation for 12 hours at 4°C resulted in further reduction in *de novo* protein synthesis (figure 6.8) such that only 15 proteins were detected above the threshold volume, 10 of which were also detected after 1.5 hours at 10°C. The level of CspA detected was similar to the level after 6 hours at 4°C, and this protein remained the most highly induced protein relative to the level detected at 37°C. One other protein (4) was induced between 2 and 10-fold. None of the proteins detected after 6 hours at 4°C were induced relative to incubation at 10°C for 1.5 hours, although protein 55 was induced between 2 and 10-fold, relative to the level detected after 2 hours at 4°C.

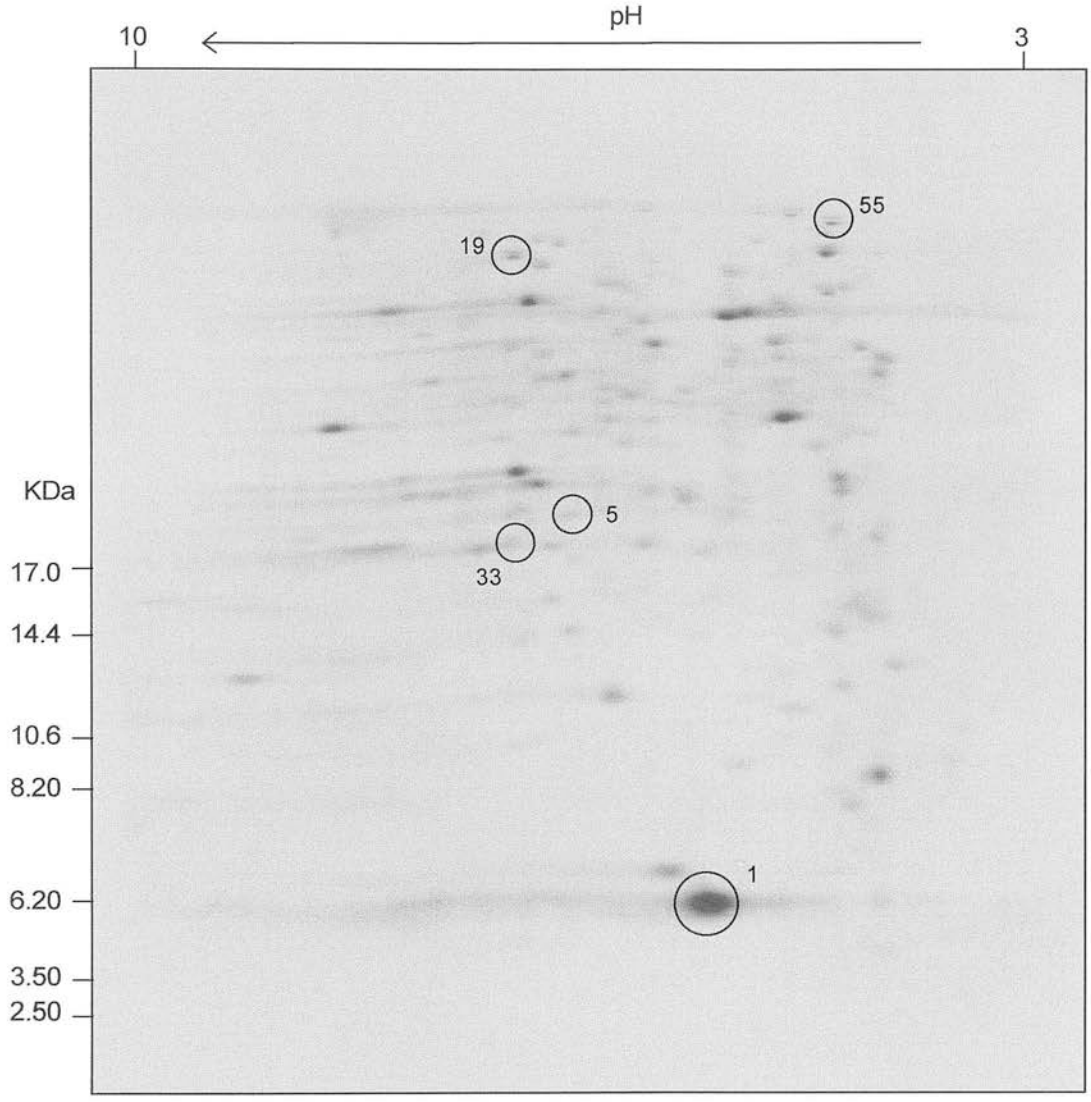


Figure 6.7 Autoradiograph of protein extracts from ^{35}S-methionine-labelled *S. typhimurium* SL1344 cells grown to mid-exponential phase at 37°C, in Spitizen medium, and incubated at 4°C for 6 hours. 3 ml of the culture was radio-actively labeled for 30 minutes at 4°C. The cells were disrupted by sonication and the protein extracts separated by 2-D PAGE as described in the legend for figure 6.1.

Protein spots whose synthesis was found to increase following a shift to 4°C are circled. These numbered proteins are listed in table 6.5.

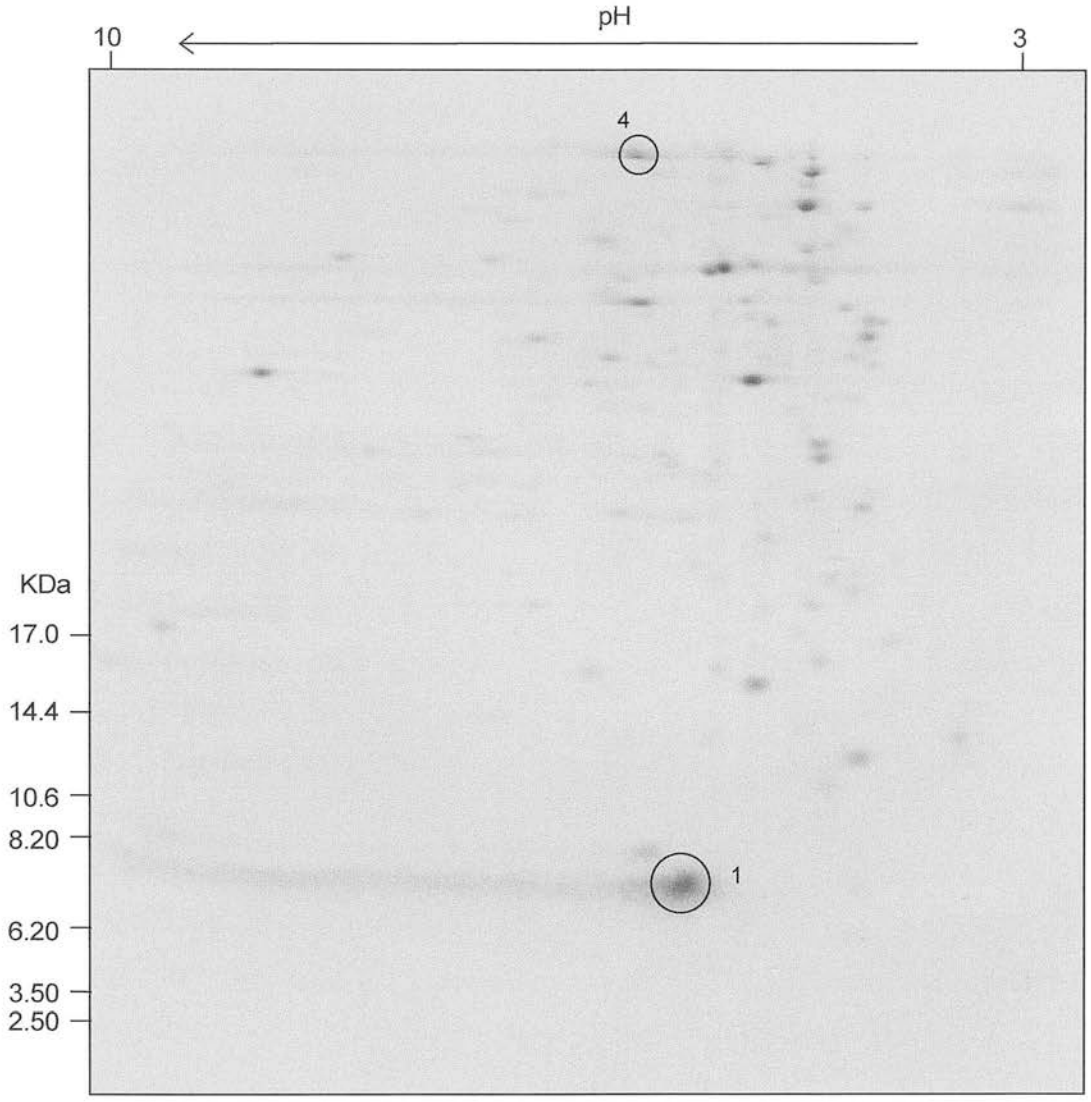


Figure 6.8 Autoradiograph of protein extracts from ^{35}S-methionine-labelled *S. typhimurium* SL1344 cells grown to mid-exponential phase at 37°C, in Spitizen medium, and incubated at 4°C for 12 hours. 3 ml of the culture was radio-actively labeled for 30 minutes at 4°C. The cells were disrupted by sonication and the protein extracts separated by 2-D PAGE as described in the legend for figure 6.1.

Protein spots whose synthesis was found to increase following a shift to 4°C are circled. These numbered proteins are listed in table 6.5.

Overall *de novo* protein synthesis increased after incubation at 4°C for 24 hours (figure 6.9), relative to that observed after 12 hours and 25 proteins were detected that were above the threshold volume. Only 9 of these were also detected after 1.5 hours at 10°C. CspA remained the most highly induced protein at this time point and was 2.5-fold higher than at 37°C. No other proteins were induced relative to the level detected prior to the temperature downshift.

Following incubation for 96 hours at 4°C, the total number of proteins detected above the threshold level increased to 57 (figure 6.10) of which 25 were also detected after incubation of 1.5 hours at 10°C. At the 96 hour time point, CspA was induced approximately 10-fold relative to the level detected at 37°C (panel B of figure 6.3). (It should be noted that due to artefacts that occurred during the gel drying process, the level of CspA detected may be an underestimation of the actual level that was present). 5 further proteins (5, 6, 7, 47, 50) were induced between 2 and 10-fold. Compared with 37°C, protein 57 (panel E, figure 6.3), was shown to be very highly induced (approximately 40-fold) following extended incubation at 4°C, and was the most abundant protein detected. This protein was also induced 4-fold relative to the level detected after 1.5 hours at 10°C. Protein 51, which was not detected at 37°C, was induced 2-fold relative to the level detected after incubation for 1.5 hours at 10°C.

In comparison to the levels detected after incubation for 1.5 hours at 10°C, 4 proteins (47, 50, 51, 57) were induced between 2 and 10-fold, after 96 hours at 4°C. Furthermore, 3 proteins (6, 7, 51) were induced between 2 and 10-fold and 1 protein (57) was induced more than 10-fold, relative to the level detected after incubation for 2 hours at 4°C.

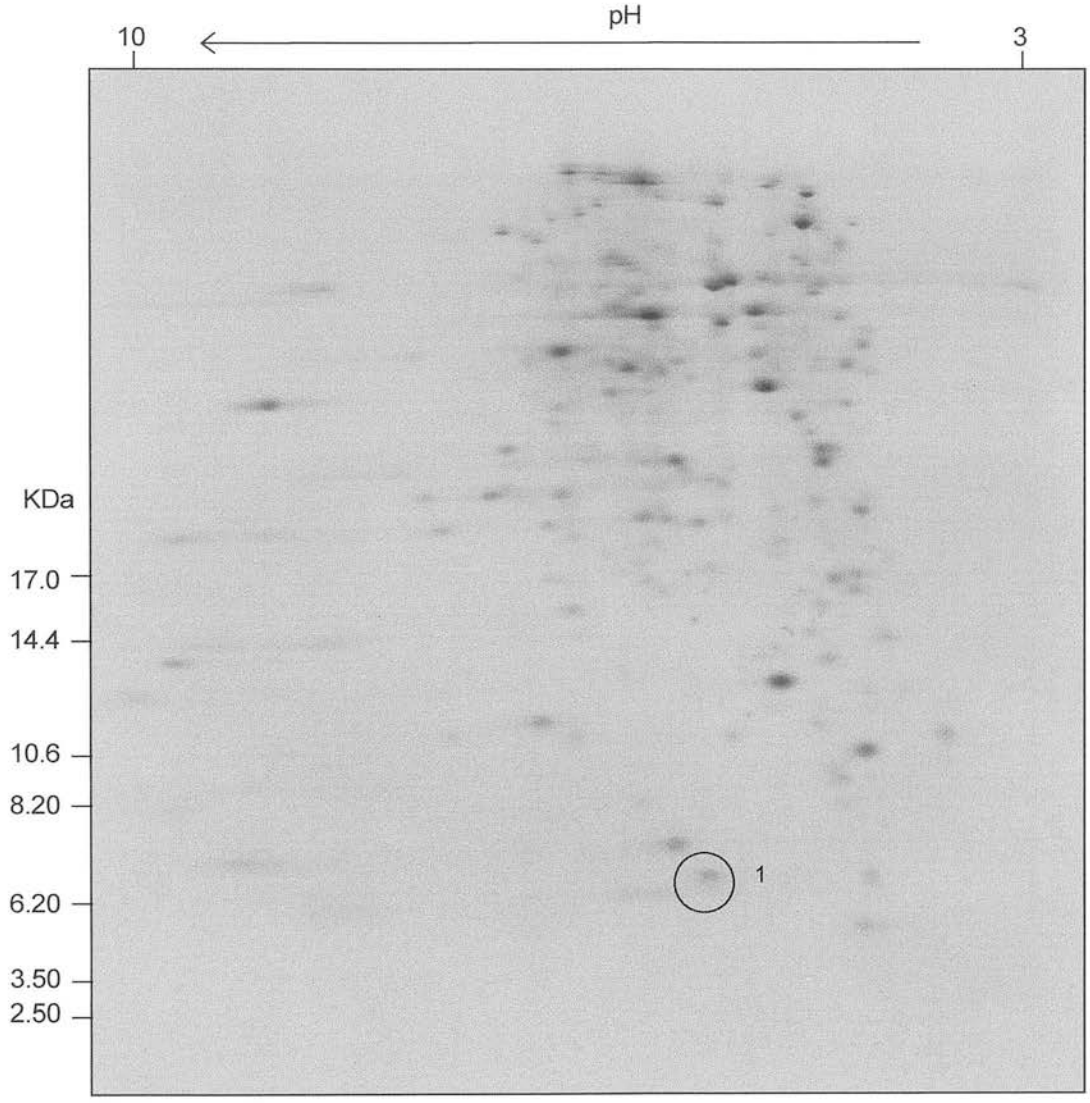


Figure 6.9 Autoradiograph of protein extracts from ^{35}S-methionine-labelled *S. typhimurium* SL1344 cells grown to mid-exponential phase at 37°C, in Spitizen medium, and incubated at 4°C for 24 hours. 3 ml of the culture was radio-actively labeled for 30 minutes at 4°C. The cells were disrupted by sonication and the protein extracts separated by 2-D PAGE as described in the legend for figure 6.1.

The protein spots whose synthesis was found to increase following a shift to 4°C is circled. This numbered protein is listed in table 6.5.

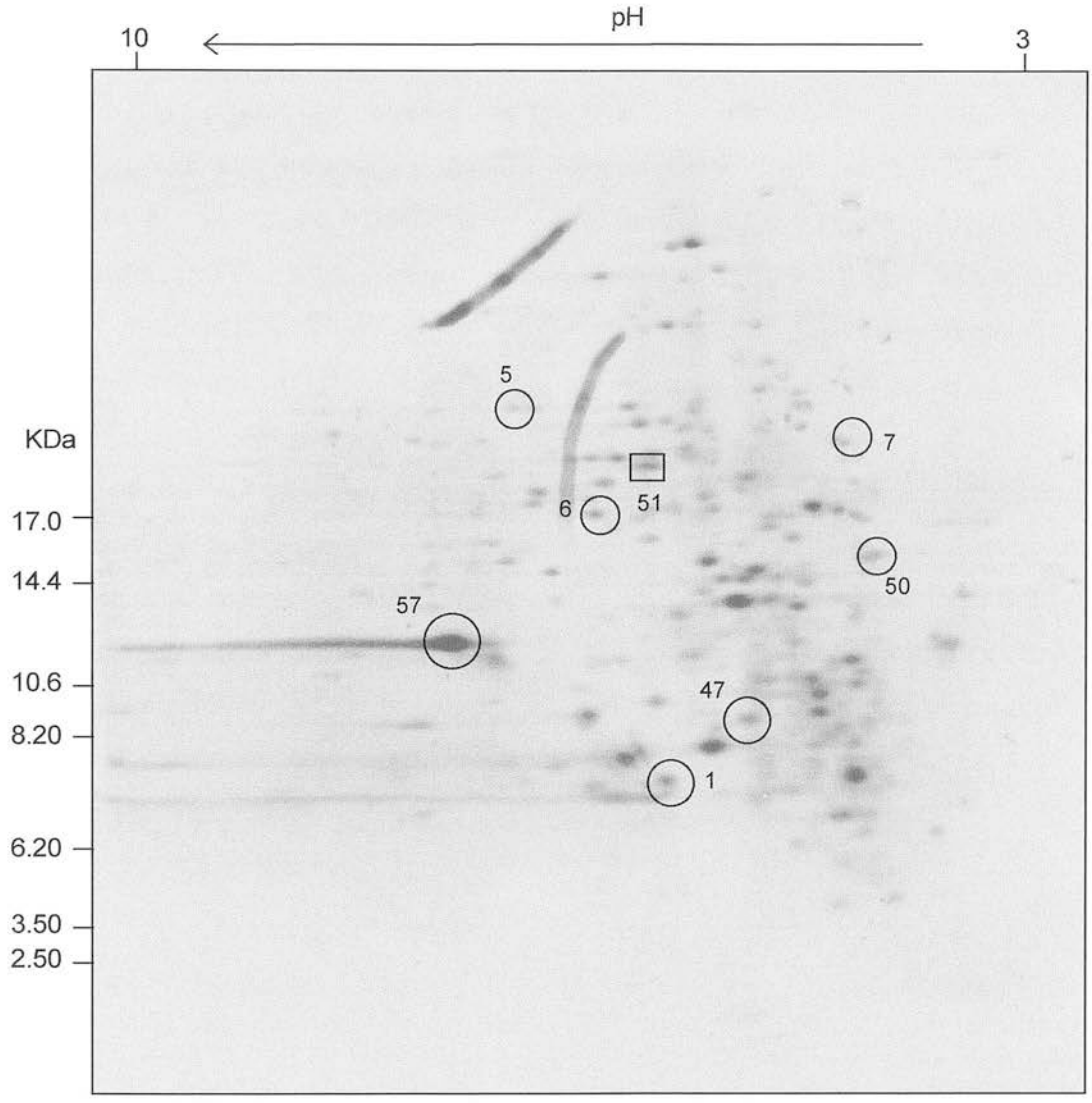


Figure 6.10 Autoradiograph of protein extracts from ^{35}S-methionine-labelled *S. typhimurium* SL1344 cells grown to mid-exponential phase at 37°C, in Spitizen medium, and incubated at 4°C for 96 hours. 3 ml of the culture was radio-actively labeled for 30 minutes at 4°C. The cells were disrupted by sonication and the protein extracts separated by 2-D PAGE as described in the legend for figure 6.1.

Protein spots whose synthesis was found to increase following a shift to 10°C are circled. The positions of the boxes represents a protein that was only detected at 4°C and was not evident at 37°C. These numbered proteins are listed in table 6.5.

As an overall summary, the proteins that were induced in an exponentially growing culture of SL1344, following a temperature shift from 37°C to 10°C or 4°C are listed in table 6.5. 13 proteins (1, 4, 5, 6, 7, 8, 14, 15, 16, 18, 19, 22, 23) were transiently induced at 10°C and are therefore termed CIPs, whereas 15 proteins (9, 10, 21, 32, 36, 38, 40, 41, 42, 43, 47, 48, 49, 54, 55) were synthesised at a high level relative to that detected at 37°C, after prolonged incubation at 10°C, and are therefore termed CAPs. 7 proteins (24, 26, 27, 28, 51, 52, 53) that were not previously detected at 37°C, were subsequently detected following incubation at 10°C or 4°C.

Several of the *S. typhimurium* proteins have been provisionally identified by comparison of their apparent molecular size and ioselectric point with known cold-induced *E. coli* proteins. These proteins are as follows: number 4 resembles SdhA, number 55 resembles Hsc66, number 20 resembles SucB, number 8 resembles CarA, number 43 resembles OmpA, number 6 resembles SodA, number 40 resembles IbpB, number 18 resembles RbfA and number 54 resembles H-NS (54) (Jones *et al.*, 1987; VanBogelen *et al.*, 1996).

Table 6.5 (on the following page) Cold shock inducible proteins detected from cultures of SL1344 that were in exponential phase prior to temperature reduction from 37°C to 10°C or 4°C. The proteins are highlighted in figures 6.1. 6.2, 6.3, 6.4, 6.5, 6.6, 6.7 and 6.8. The apparent isoeletric point and molecular weights of the proteins were calculated from a set of 2-D standards, described in chapter 2 of this thesis. The relative levels of abundance have been given for the proteins detected after 1.5 and 4 hours of incubation at 10°C, and for 2 and 96 hours of incubation at 4°C.

Symbols and abbreviations for table 6.5:
>: the level of induction was between 2 and 10-fold relative to the pre-shift levels detected.
>>: the level of induction was greater than 10-fold relative to the pre-shift levels detected.
<: the level of protein detected was repressed relative to the pre-shift levels.
-: the level of protein detected was less than 2-fold different relative to the pre-shift level.
LTP (low temperature protein): the protein was not detected prior to the temperature reduction.
nd: the protein was not detected.
nc: there was no change in the level of protein detected

Protein number	pI	MW (KDa)	Incubation details 1.5 hours, 10°C	4 hours, 10°C	2 hours, 4°C	96 hours, 4°C
1	5.99	7.17	>>	>>	>>	>
2	5.17	6.72	>>	nd	>>	<
3	6.49	77.45	>>	>>	nd	nd
4	6.06	65.77	>>	-	nd	nd
5	7.25	28.05	>>	nc	>	>
6	6.43	23.12	>>	>>	<	>
7	5.08	24.21	>		-	>
8	5.9	40.84	>	>	<	nd
9	5.62	25.02	>	>	>	-
10	8.1	23.37	>	>>	nd	<
11	6.38	65.24	>	>>	nd	nd
13	7.32	46.29	>	nd	nd	nd
14	8.75	5.13	>	>	<	-
15	8.47	7.53	>	<	>	nd
16	7.31	6.33	>	nd	nd	>
17	7.34	19.85	>	<	-	nd
18	6.66	13.67	>	-	>	-
19	7.27	56.63	>	>	<	nd
20	6.4	47.06	>	>	<	nd
21	6.5	37.54	>	>>	<	nd
22	7.37	49.96	>	nd	-	nd
23	7.64	32.94	>	>	-	<
24	7.07	56.47	LTP	nc	nd	nd
26	6.24	42.31	LTP	<	<	nd
27	9.35	8.22	LTP	<	nc	nd
28	9.92	16.05	LTP	nc	nc	nd
32	4.75	23.37	-	>>	-	nd
33	7.47	25.43	>	>>	nd	nd
34	5.31	31.28	-	>>	>>	-
35	6.28	63.49	-	>>	nd	nd
36	7.24	33.21	>	>	nd	nd
37	6.53	68.52	-	>	nd	nd
38	5.86	19.37	<	>	nd	-
39	6.87	73.34	<	>	nd	nd
40	4.83	18.05	-	>	-	-
41	6.64	34.59	-	>	<	nd
42	6.74	19.74	-	>	-	nd
43	5.53	26.64	-	>	<	<
44	5.34	23.63	>	>	<	<
45	5.4	48.62	<	>	<	nd
46	6.36	63.14	<	>	nd	nd
47	5.24	9.57	-	>	-	>
48	8.87	35.84	-	>	<	nd
49	7.2	39.42	<	>	nd	nd
50	4.68	17.47	-	<	nd	>
51	6.1	25.71	LTP	nc	nc	>
52	8.97	23.76	LTP	>	nd	nd
53	7.76	13.86	LTP	>	nd	nd
54	5.72	13.6	<	-	<	<
55	5.4	62.46	-	<	<	<
57	7354	11.67	>	>	nc	>>

6.2.3 Protein expression in stationary phase SL1344 cells following a shift from 37°C to 10°C.

The effect of growth phase on the cold shock response has not been investigated in detail at molecular level. Gram negative bacteria that are in stationary phase are known to be very resistant to environmental stresses (Kolter *et al.*, 1993). In order to address whether stationary phase cells of *S. typhimurium* were capable of initiating a cold shock response, their capacity for *de novo* protein synthesis was investigated, both above and below the minimum temperature permissible for growth, using 2-D PAGE.

S. typhimurium SL1344 cells were grown in defined Spitzizen media for 24 hours at 37°C, to stationary phase. 3ml aliquots were either incubated at 37°C or shifted to 10°C or 4°C. The cells were incubated with 24 μCi of $^{35}S^{met/cys}$ described previously. Immediately following the period of radioactive labelling, the cells were disrupted by sonication and the soluble proteins from the whole cell extract were resolved by 2-D PAGE.

The numbers of proteins synthesised at each of the time points for stationary phase SL1344 at 37°C, 10°C and 4°C have been indicated (table 6.6).

Conditions	Total number of proteins above threshold level
37°C 0 hours	49
10°C 1.5 hours	17
10°C 4 hours	9
4°C 2 hours	15
4°C 6 hours	6
4°C 12 hours	3
4°C 24 hours	2
4°C 96 hours	0

Table 6.6 The number of proteins that were detected from stationary phase SL1344 cells at 37°C, 10°C and 4°C. The total number of proteins that lie above the threshold protein spot volume of 900 units has been indicated.

Incubation of a stationary phase culture of SL1344 at 37°C led to *de novo* protein synthesis of proteins with a wide range of pI and molecular sizes (figure 6.11). Overall, it is clear that as the incubation time increased at low temperatures, the number of proteins detected above the threshold level decreased. At 37°C 49 proteins were detected (table 6.6), of which 34 were also detected after 1.5 hours at 10°C. 12 of these proteins were found to be at least 2-fold more abundant at 37°C and 20 proteins were more than 10-fold more abundant at 37°C. Notably, CspA was absent in the stationary phase culture, at 37°C.

2 highly abundant proteins detected at 37°C, proteins 62 and 68, were later shown to be repressed following incubation at 10°C or 4°C (figure 6.11 and figure 6.12). The molecular weight and pI of these proteins suggest that either one may possibly be members of the CspA family. CspD, has been detected in stationary phase cultures of *E. coli* at 37°C and 1 of these could represent an equivalent in *S. typhimurium* (Yamanaka & Inouye, 1997).

Shifting the stationary phase culture of SL1344 from 37°C to 10°C for 1.5 hours, resulted in a decrease in overall protein synthesis (figure 6.13). The majority of proteins detected at 37°C were repressed at 10°C and only 17 proteins were detected above the threshold level. However, 2 proteins (48, 58) were induced more than 2-fold at 10°C, relative to their level detected at 37°C and 2 proteins (1, 2) present at 10°C were not detected at 37°C. CspA was the most abundant protein after 1.5 hours at 10°C (shown in figure 6.14, panel A).

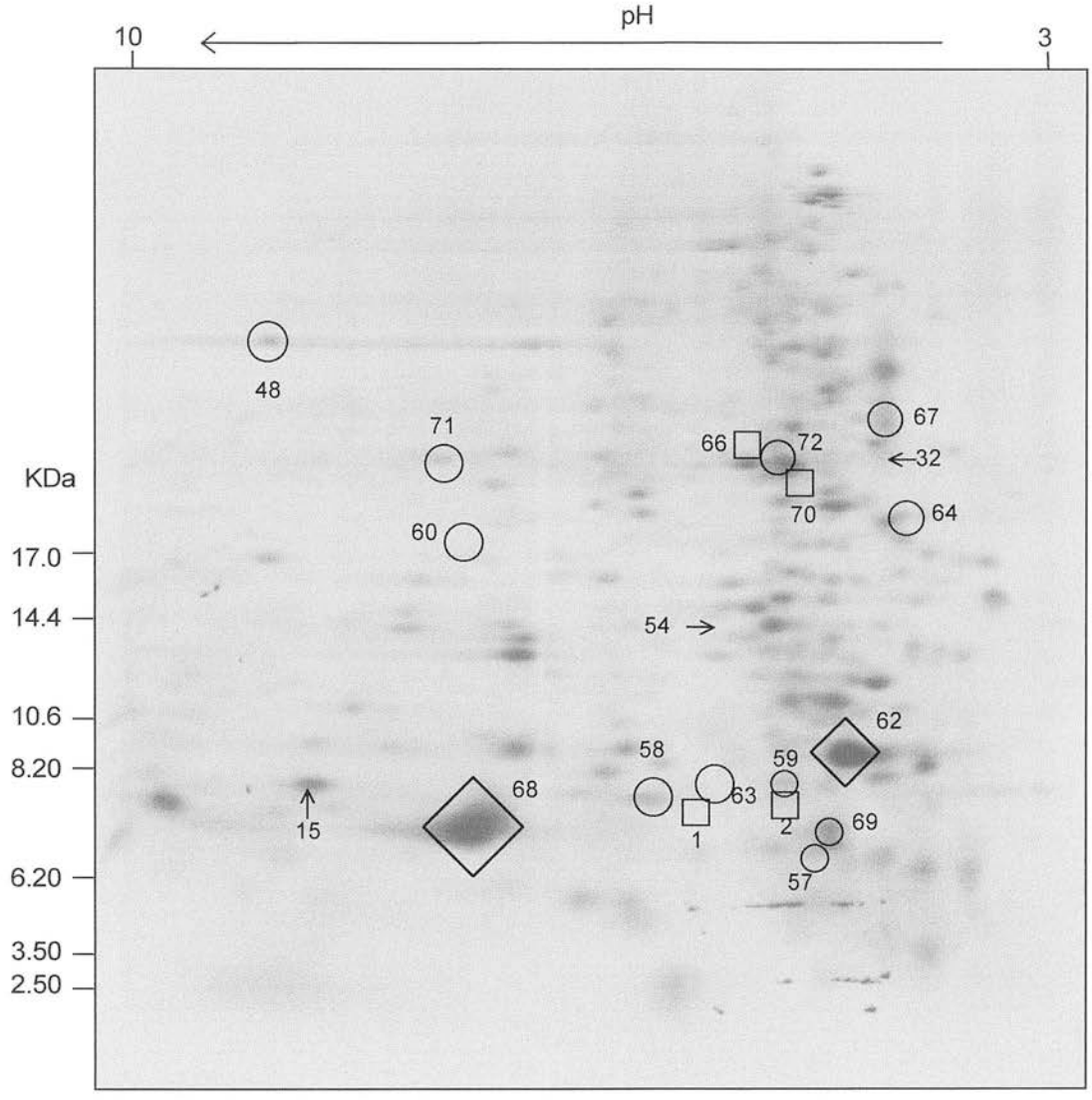


Figure 6.11 Autoradiograph of protein extracts from ^{35}S-methionine-labelled *S. typhimurium* SL1344 cells grown to stationary phase at 37°C, in Spitizen medium. 3 ml of the culture was radioactively labeled for 10 minutes at 37°C. The cells were disrupted by sonication and the protein extracts separated by 2-D PAGE, as described in the legend for figure 6.1.

Protein spots whose synthesis was found to increase following a shift to 10°C or 4°C are circled. The positions of the boxes represent proteins that were only detected at low temperatures and were not evident at 37°C. Proteins that were found to be highly induced at 37°C and repressed at 10°C or 4°C are enclosed in diamond shapes. The numbered proteins are listed in table 6.7. Other proteins of interest have been indicated with an arrow.

Panel A
Protein 62

Panel B
Protein 68

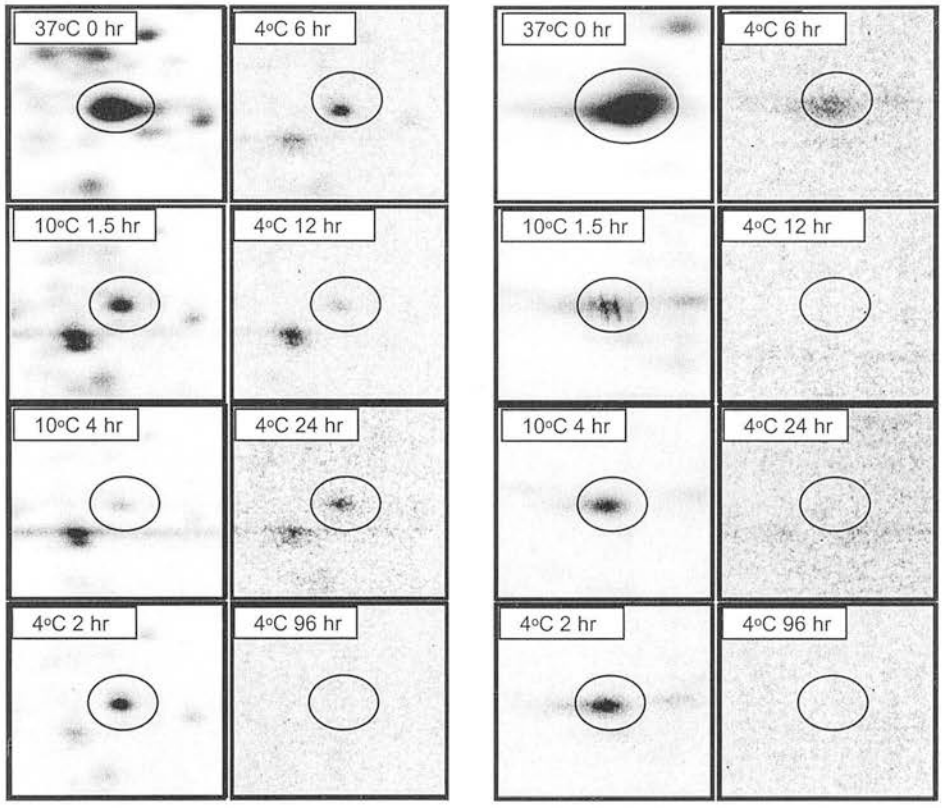


Figure 6.12

Montages showing proteins that were induced to a high level at 37°C and subsequently repressed at low temperatures. Panel A, Protein 62; panel B, Protein 68.

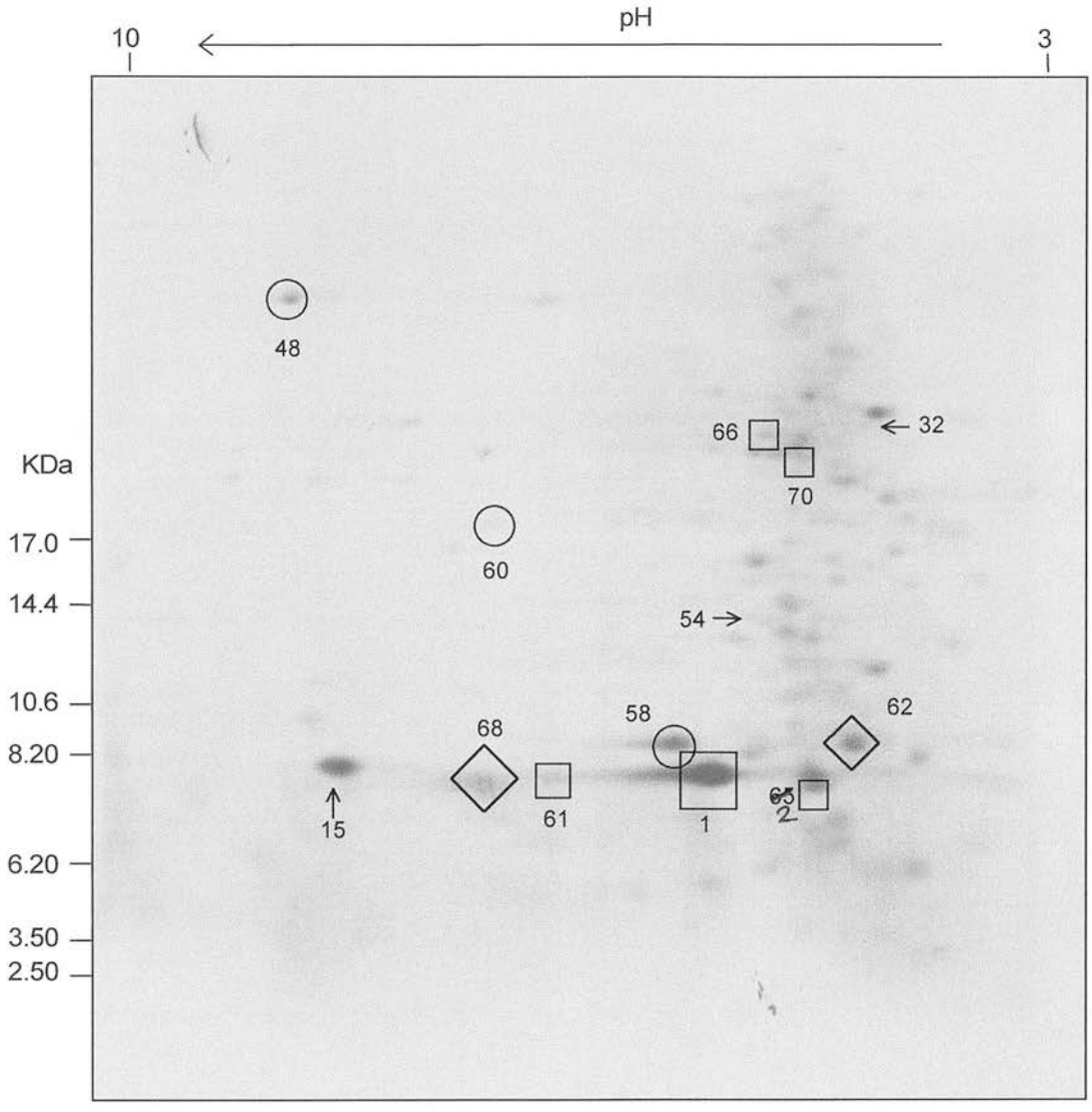


Figure 6.13 Autoradiograph of protein extracts from ^{35}S-methionine-labelled *S. typhimurium* SL1344 cells grown to stationary phase at 37°C, in Spitizen medium, and incubated at 10°C for 1.5 hours. 3 ml of the culture was radio-actively labeled for 60 minutes at 10°C. The cells were disrupted by sonication and the protein extracts separated by 2-D PAGE, as described in the legend for figure 6.1.

Protein spots whose synthesis was found to increase following a shift to 10°C are circled. The boxed proteins were only detected at 10°C and were not evident at 37°C. The numbered proteins are listed in table 6.6. Other proteins of interest have been indicated with an arrow.

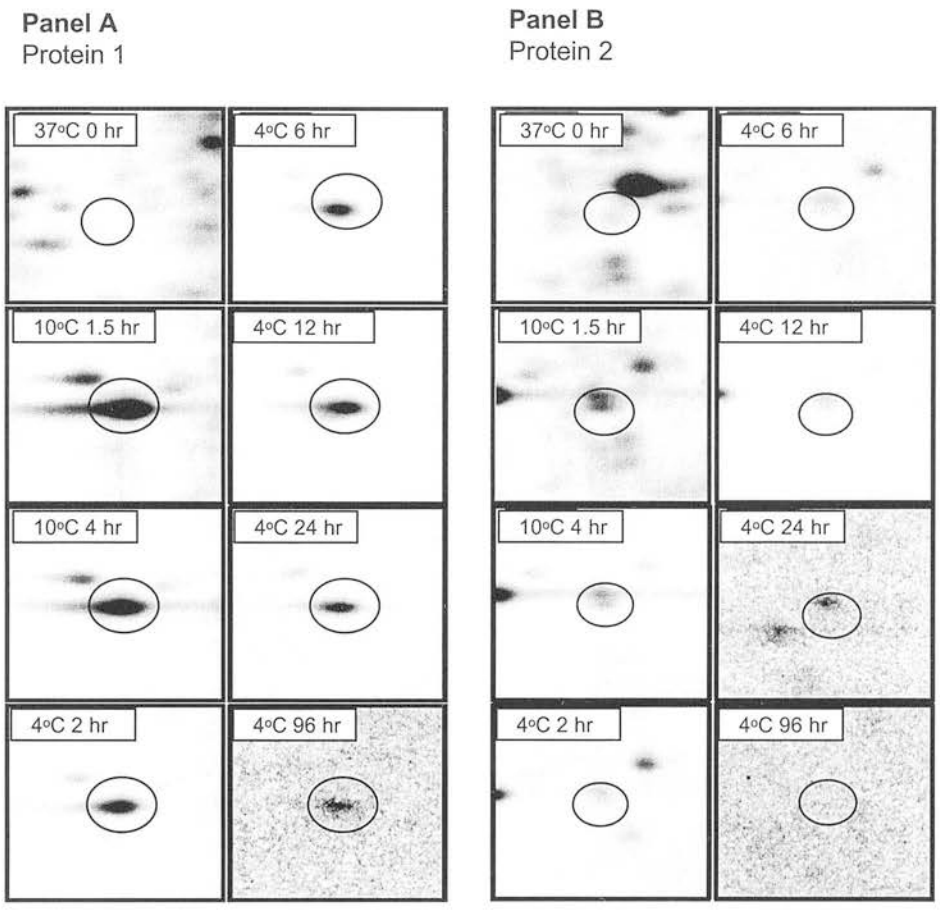


Figure 6.14

Montage showing cold-induced proteins in stationary phase SL1344 cells, following incubation at 10°C or 4°C. Panel A, Protein 1 (CspA); panel B, Protein 2.

Panel C
Protein 58

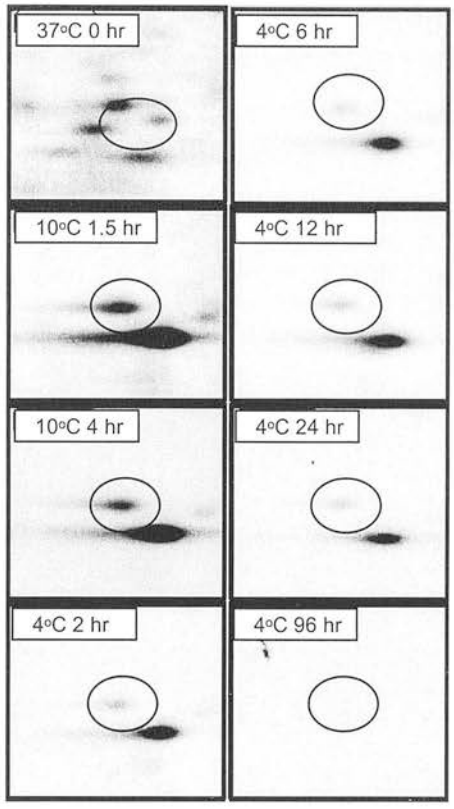


Figure 6. 14 continued

Montage showing cold-induced proteins in stationary phase SL1344 cells, following incubation at 10°C or 4°C. Panel C, Protein 58

Incubation of stationary phase cells of SL1344 for 4 hours at 10°C resulted in a further reduction of overall protein synthesis (figure 6.15), and only 9 proteins were detected above the threshold volume. 3 proteins (numbers 58, 59, 63) were induced more than 10-fold relative to the level detected at 37°C, see figure 6.14, panel C, for an example. In addition, protein 2 (figure 6.14 panel B), which was undetected at 37°C, was synthesised at a maximum level after 4 hours at 10°C. CspA was the most abundant protein at this time point and was approximately 1.5-fold more abundant than observed after 1.5 hours at 10°C.

All the proteins detected after 4 hours at 10°C matched proteins detected after 1.5 hours at 10°C. 1 protein (63) was induced between 2 and 10-fold and 1 protein (59) was induced more than 10-fold, relative to the level after 1.5 hours at 10°C.

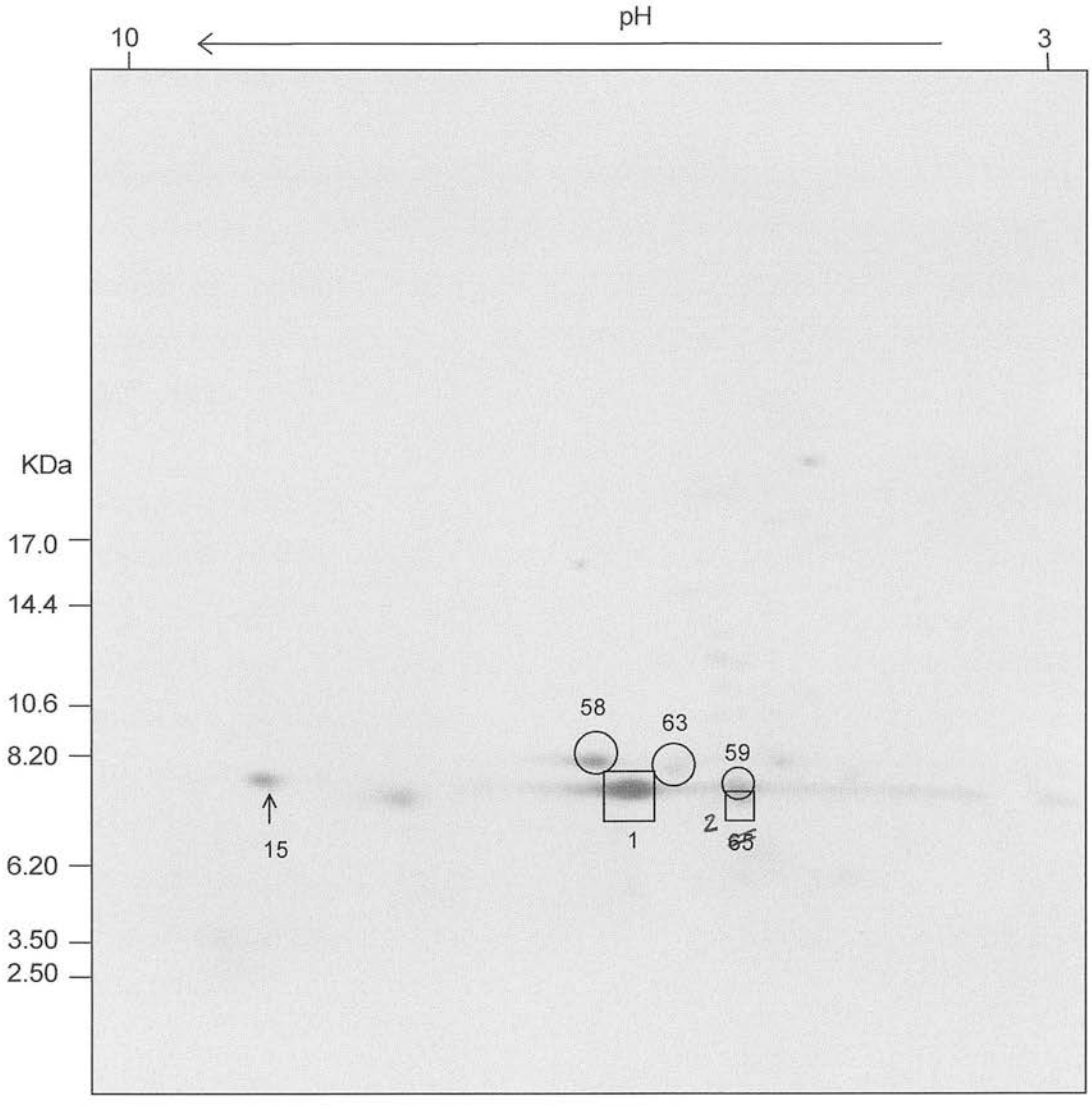


Figure 6.15 Autoradiograph of protein extracts from ^{35}S-methionine-labelled *S. typhimurium* SL1344 cells grown to stationary phase at 37°C, in Spitizen medium, and incubated at 10°C for 4 hours. 3 ml of the culture was radio-actively labeled for 60 minutes at 10°C. The cells were disrupted by sonication and the protein extracts separated by 2-D PAGE, as described in the legend for figure 6.1.

Protein spots whose synthesis was found to increase following a shift to 10°C are circled. The boxed proteins were only detected at 10°C and were not evident at 37°C. The numbered proteins are listed in table 6.6. Other proteins of interest have been indicated with an arrow.

6.2.4 Protein expression in stationary phase SL1344 cells following a shift from 37°C to 4°C.

De novo protein synthesis was also investigated in stationary phase SL1344 bacteria that were incubated at 4°C. These conditions were thought to mimic those that *S. typhimurium* may encounter when associated with food-stuffs that are refrigerated. The cultures were treated as described previously and the soluble proteins from the whole cell extracts resolved by 2-D PAGE.

Incubation of stationary phase SL1344 cells for 2 hours at 4°C resulted in a decrease in overall protein synthesis (figure 6.16). Only 15 proteins were detected above the threshold volume, all of which were also detected after 1.5 hours at 10°C. CspA was the most highly induced protein at this temperature (figure 6.14, panel A). The level of synthesis of 4 proteins, 58 (figure 6.14, panel C), 59, 60 and 64, increased between 2 and 10-fold, while 8 proteins were repressed, relative to the level detected at 37°C. 3 proteins (72, 59, 69) were induced between 2 and 10-fold and 1 proteins (60) was induced more than 10-fold, relative to the level detected after 1.5 hours at 10°C. Synthesis of the CspD-like proteins (proteins 62 and 68) were highly repressed relative to 37°C.

Incubation of stationary phase SL1344 for 6 hours at 4°C resulted in a further decrease in overall *de novo* protein synthesis (figure 6.17). Only 6 proteins above the threshold volume were detected, all of which were also detected after 1.5 hours at 10°C (table 6.6). Again, CspA was the most abundant protein (figure 6.14 panel A).

Further decrease in overall *de novo* protein synthesis was observed following 12 hour incubation of stationary phase SL1344 at 4°C (figure 6.18), so that only 2 proteins (58, CspA) above the threshold level were detected (figure 6.14, panel A and panel C, respectively). Moreover, after 24 hours at 4°C (figure 6.19), only 2 proteins (CspA, 58) were detected with a spot volume greater than 900. By 96 hours, CspA was the only detectable protein and no proteins were detected with a spot volume greater than 900 units (figure 6.20).

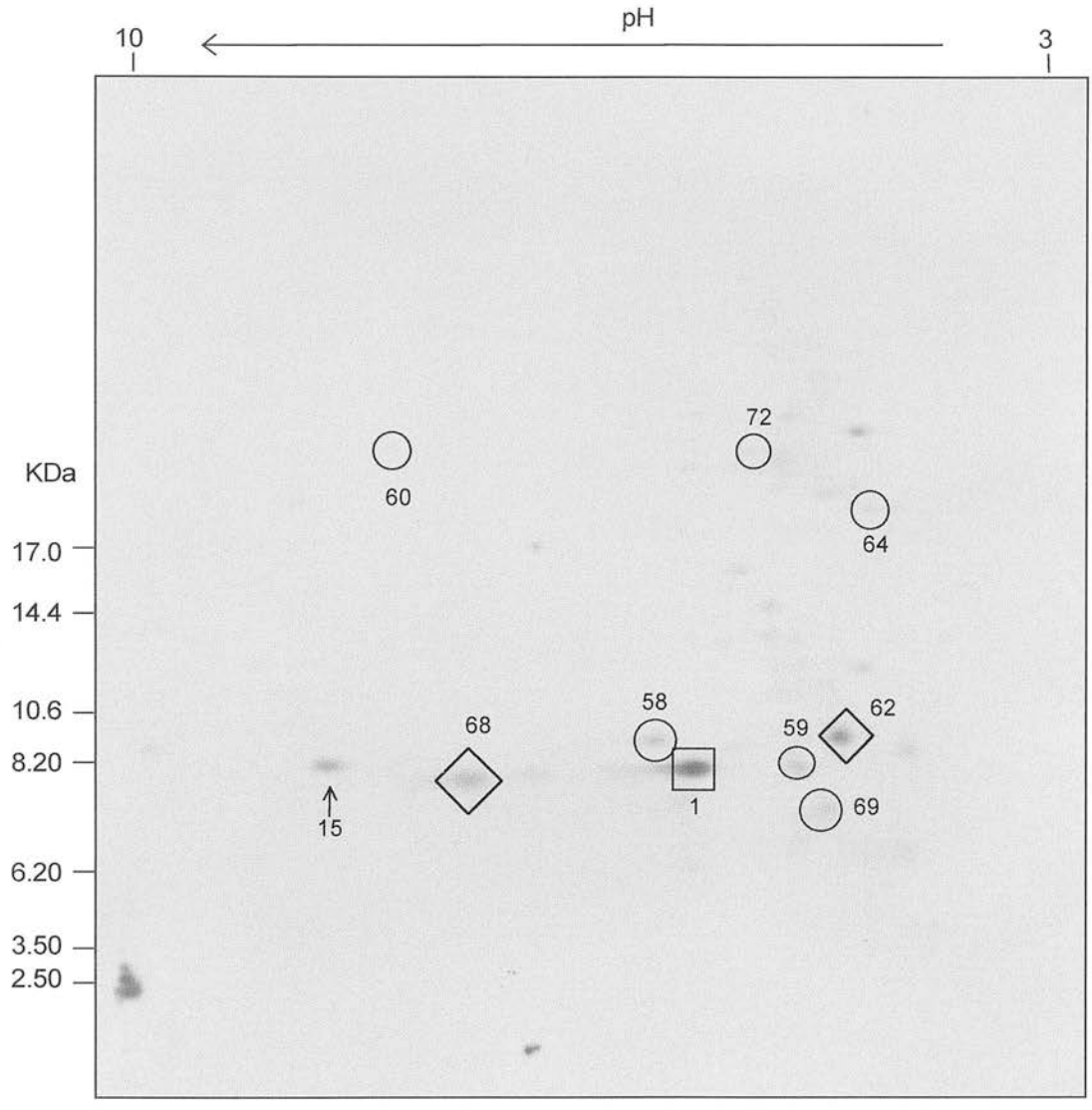


Figure 6.16 Autoradiograph of protein extracts from ^{35}S-methionine-labelled *S. typhimurium* SL1344 cells grown to stationary phase at 37°C, in Spitizen medium, and incubated at 4°C for 2 hours. 3 ml of the culture was radio-actively labeled for 60 minutes at 4°C. The cells were disrupted by sonication and the protein extracts separated by 2-D PAGE, as described in the legend for figure 6.1.

Protein spots whose synthesis was found to increase following a shift to 4°C are circled. The boxed protein was only detected at 4°C and was not evident at 37°C. The numbered proteins are listed in table 6.6. Other proteins of interest have been indicated with an arrow.

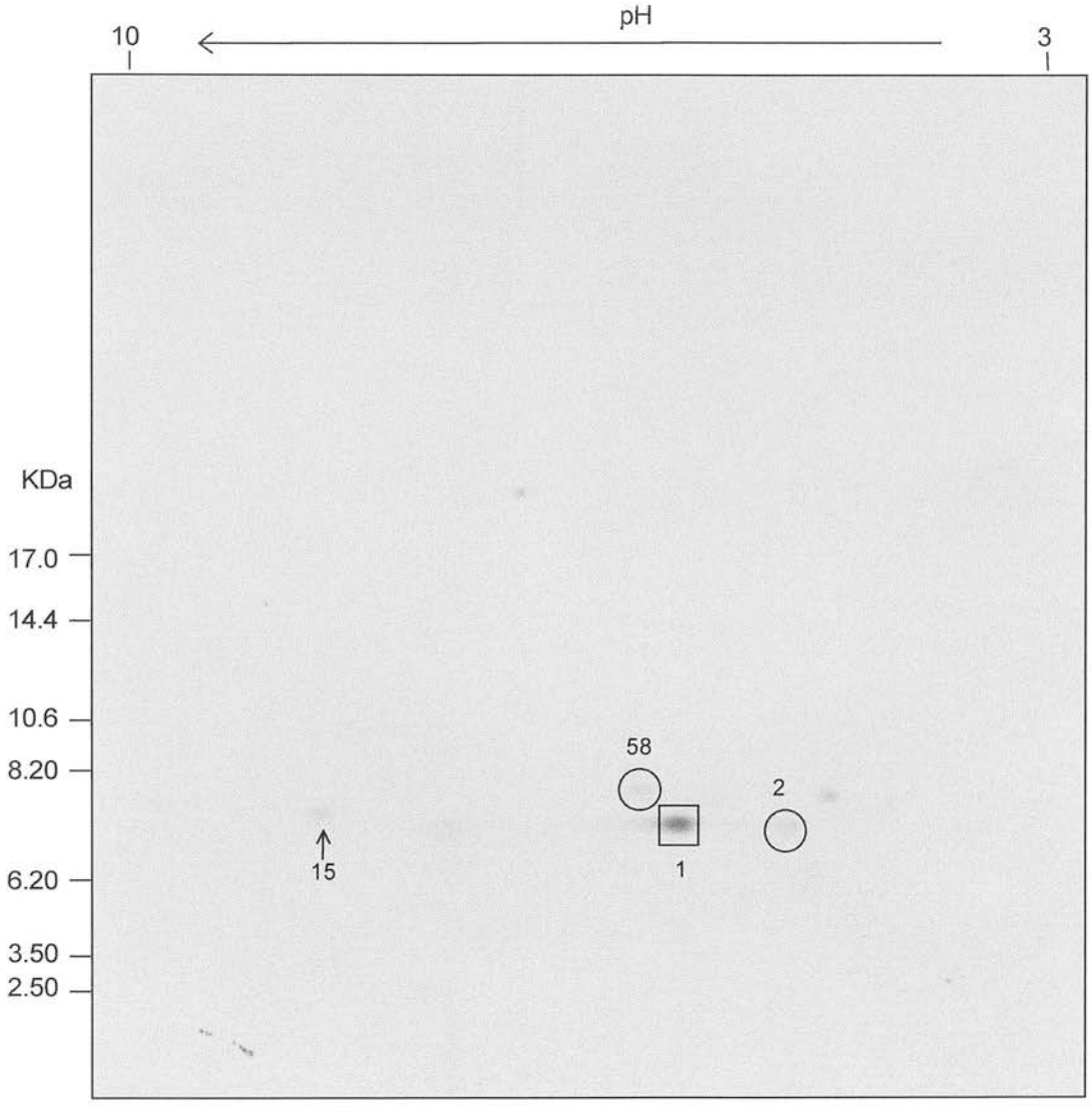


Figure 6.17 Autoradiograph of protein extracts from ^{35}S-methionine-labelled *S. typhimurium* SL1344 cells grown to stationary phase at 37°C, in Spitizen medium, and incubated at 4°C for 6 hours. 3 ml of the culture was radio-actively labelled for 60 minutes at 4°C. The cells were disrupted by sonication and the protein extracts separated by 2-D PAGE, as described in the legend for figure 6.1.

The boxed protein was only detected at 4°C and was not evident at 37°C. This numbered protein is listed in table 6.6. Other proteins of interest have been indicated with an arrow.

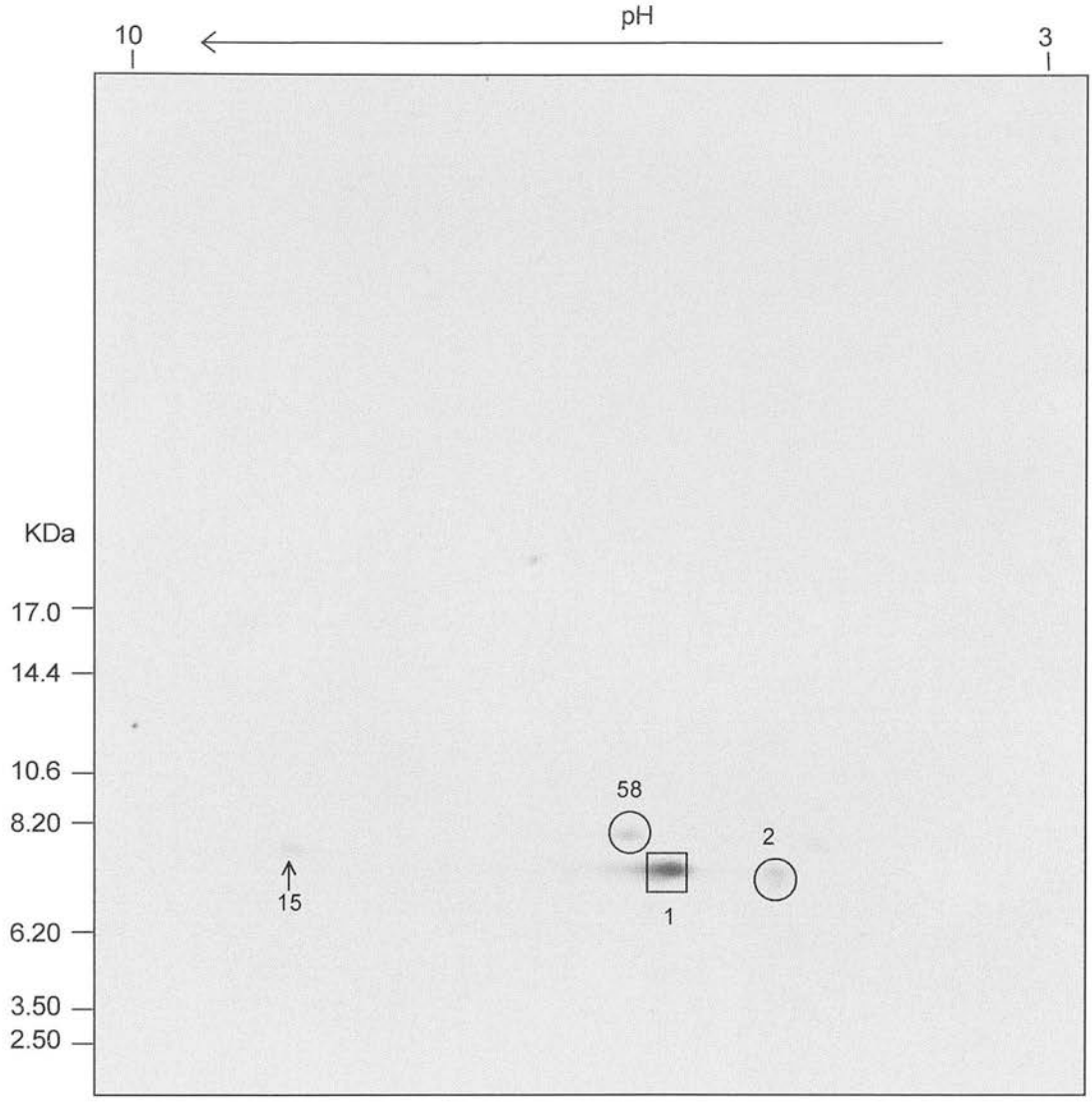


Figure 6.18 Autoradiograph of protein extracts from ^{35}S-methionine-labelled *S. typhimurium* SL1344 cells grown to stationary phase at 37°C, in Spitizen medium, and incubated at 4°C for 12 hours. 3 ml of the culture was radio-actively labelled for 60 minutes at 4°C. The cells were disrupted by sonication and the protein extracts separated by 2-D PAGE, as described in the legend for figure 6.1.

Protein spots whose synthesis was found to increase following a shift to 4°C are circled. The boxed protein was only detected at 4°C and was not evident at 37°C. The numbered proteins are listed in table 6.6. Other proteins of interest have been indicated with an arrow.

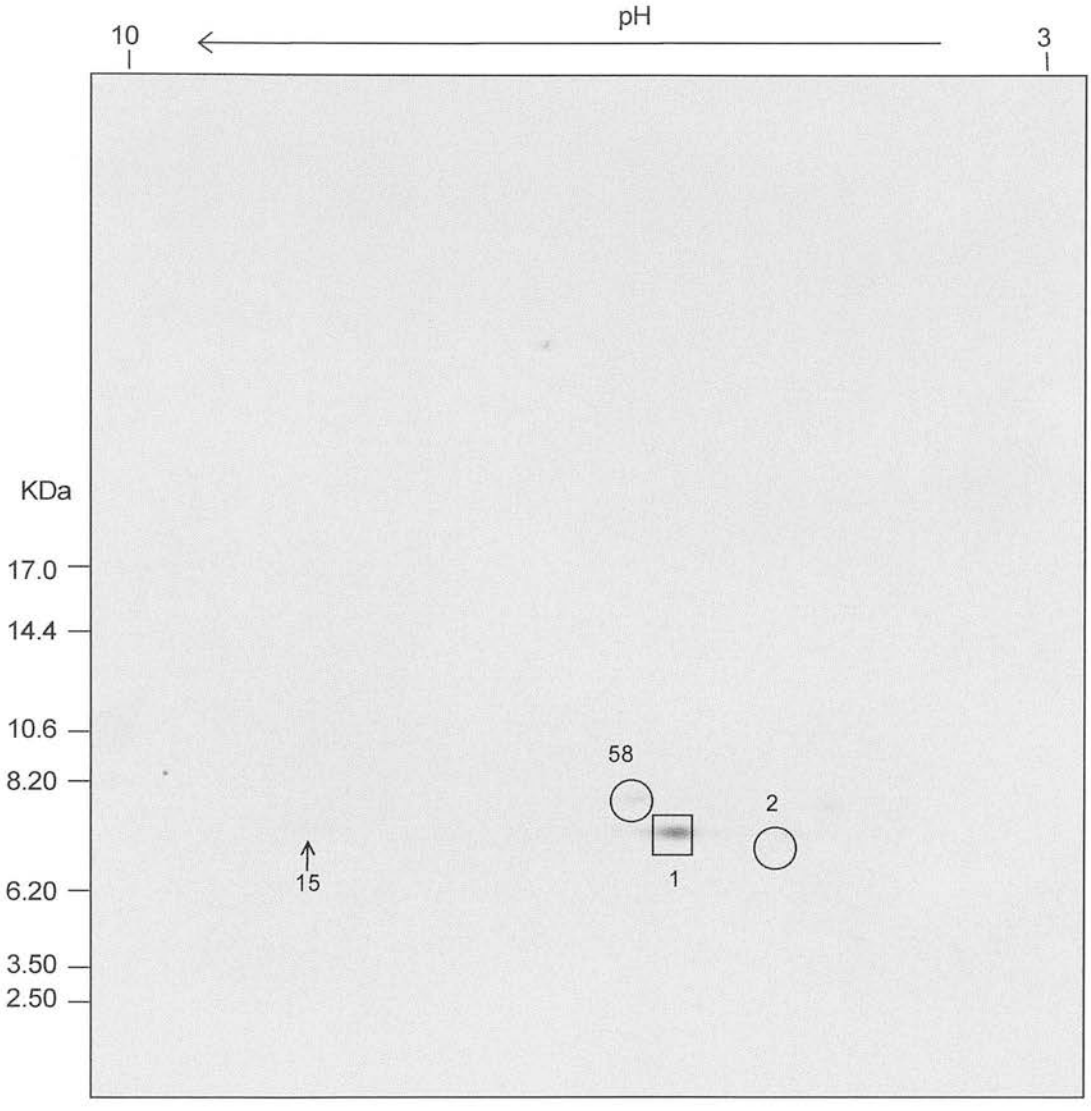


Figure 6.19 Autoradiograph of protein extracts from ^{35}S-methionine-labelled *S. typhimurium* SL1344 cells grown to stationary phase at 37°C, in Spitizen medium, and incubated at 4°C for 24 hours. 3 ml of the culture was radio-actively labelled for 60 minutes at 4°C. The cells were disrupted by sonication and the protein extracts separated by 2-D PAGE, as described in the legend for figure 6.1.

Protein spots whose synthesis was found to increase following a shift to 4°C are circled. The boxed protein was only detected at 4°C and was not evident at 37°C. The numbered proteins are listed in table 6.6. Other proteins of interest have been indicated with an arrow.

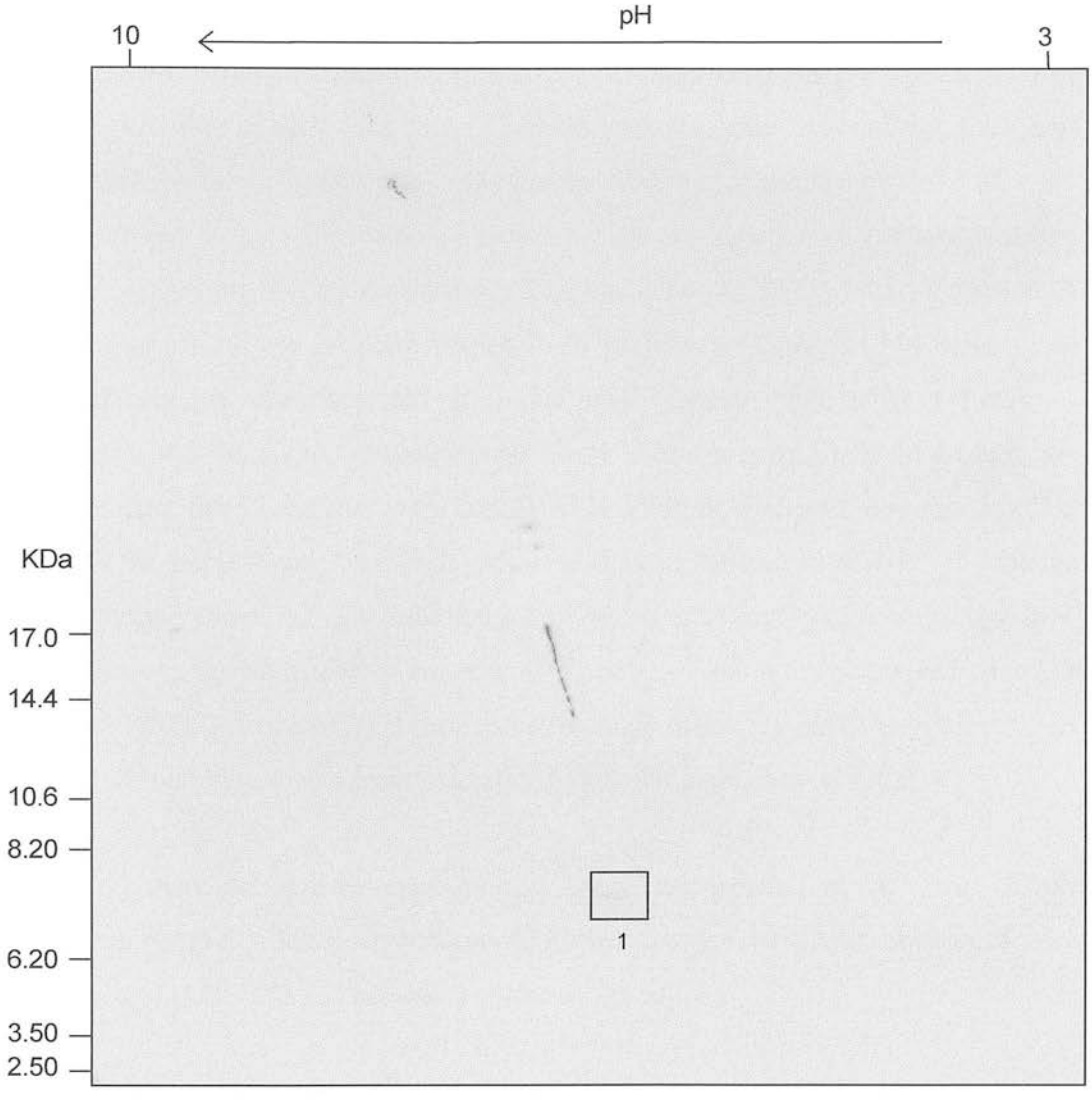


Figure 6.20 Autoradiograph of protein extracts from ^{35}S-methionine-labelled *S. typhimurium* SL1344 cells grown to stationary phase at 37°C, in Spitizen medium, and incubated at 4°C for 96 hours. 3 ml of the culture was radio-actively labelled for 60 minutes at 4°C. The cells were disrupted by sonication and the protein extracts separated by 2-D PAGE, as described in the legend for figure 6.1.

The boxed protein was only detected at 4°C and was not evident at 37°C. This numbered protein is listed in table 6.6.

Overall, it can be seen that shifting a stationary phase culture of SL1344 from 37°C to either 10°C or 4°C resulted in a similar pattern of reduction in the overall level of protein synthesis in each case, and coincided with increased production of a set of cold shock proteins. Contrary to observations from exponentially growing SL1344 cells, different sets of cold induced proteins were not induced in a time dependent manner, suggesting that an acclimation phase had occurred before initial induction of proteins as part of the adaptive response. In stationary phase SL1344 cells, cold-induced proteins were detected at a marginally higher level after 4 hours of incubation at 10°C. CspA (protein 1) was found to be the most abundant protein that was detected from cells that were incubated at 10°C or 4°C, and was not detected prior to the temperature downshift. After prolonged incubation at 10°C 9 proteins were detected above the threshold level, 3 of which were highly expressed. (No new proteins were detected after 4 hours at 10°C which were not synthesised after 1.5 hours at 10°C). In contrast, 4 proteins were induced following incubation for 2 hours at 4°C, but only CspA was detectable after prolonged incubation at 4°C.

Table 6.7 lists the protein numbers that were cold-induced at 10°C or 4°C in stationary phase SL1344 cells. Proteins 62 and 68 are also listed since they were very highly expressed 37°C and repressed at low temperatures.

Table 6.7 Cold shock inducible proteins detected from cultures of SL1344 that were in stationary phase prior to temperature reduction from 37°C to 10°C or 4°C. The proteins are highlighted in figures 6.9. 6.10, 6.11, 6.12, 6.13, 6.14, 6.15 or 6.16. The apparent isoeletric point and molecular weights of the proteins were calculated from a set of 2-D standards, described in chapter 2 of this thesis. The relative levels of abundance have been given for the proteins detected after 1.5 and 4 hours of incubation at 10°C, and for 2 and 96 hours of incubation at 4°C. The abbreviations and symbols are as described for table 6.5.

Protein number	pI	MW (KDa)	Incubation details			
			1.5 hours, 10°C	4 hours, 10°C	2 hours, 4°C	96 hours, 4°C
1	5.42	7.14	LTP	nc	nc	<
2	4.59	6.86	LTP	nc	<	nd
15	8.02	7.25	nc	nc	nc	nd
32	4.13	20.73	LTP	<	<	nd
48	8.36	31.28	>	<	nd	nd
58	5.63	7.89	>	>>	>	nd
59	4.65	7.18	-	>>	>	nd
60	6.93	15.32	<	nd	>	nd
62	4.34	7.81	<	<	<	nd
63	5.07	7.58	>	>>		nd
64	4.08	19.1	<	-	>	nd
66	4.95	20.42	LTP	nd	nc	nd
67	4.46	24.22	-	-	nd	nd
68	7	6.9	<	<	<	nd
68	5.04	6.45	<	-	nd	nd
69	4.44	6.16	<		-	nd
70	4.68	18.87	LTP	nd	>	nd
71	7.36	21.18	<	nd	nd	nd
72	4.74	19.63	<	<	<	nd

6.2.5 Protein expression in exponentially growing MPG361 (*cspB*::Mu*dlux*) following a shift from 30°C to 10°C.

Previous studies with both *B. subtilis* (Graumann *et al.*, 1997) and *E. coli* (Bae *et al.*, 1997) have suggested that mutation of members of the CspA family can lead to pleiotrophic effects in protein synthesis at low temperatures. In addition, it has been shown that the levels of remaining members of the CspA family may increase in the absence of the major cold shock protein. Thus, the effect of mutation of *S. typhimurium cspB* (*cspB*::Mu*dlux*) on cold shock protein induction was examined in strain MPG361 to assess how it affected protein expression. Exponentially growing MPG361 cells were shifted from 30°C to 10°C for 1.5 and 4 hours. 30°C was chosen as the pre-shift temperature because cold shock induction of *cspB* had been investigated previously in strain MPG361 (see chapter 4 of this thesis, Craig *et al.*, 1998). In this strain, a putative fusion protein of 71 amino acids is formed between the truncated CspB and the end of the Mu*dlux* element, following cold shock, with a molecular size of 8.0 KDa and isolelectic point of 9.9 (Francis, 1994).

The cells were grown to mid-exponential phase at 30°C, as described previously. 3ml aliquots were either maintained at 30°C or shifted 10°C and with 24 μCi $^{35}S^{met/cys}$ as described previously. Whole cell extracts were separated by 2-D PAGE, described previously. Table 6.8 lists the numbers of proteins detected at each of the time points.

Conditions	Number of proteins above threshold
30°C 0 hours	159
10°C 1.5 hours	36
10°C 4 hours	93

Table 6.8 The number of proteins that were detected from exponentially growing MPG361 detected at 30°C and 10°C. The total number of proteins that lie above the threshold protein spot volume of 900 units.

De novo protein synthesis of exponentially growing MPG361 cells at 30°C is shown in figure 6.21. 159 proteins were detected above the threshold volume over a pI range of 3 – 10 and between 2.5 KDa and 70 KDa.

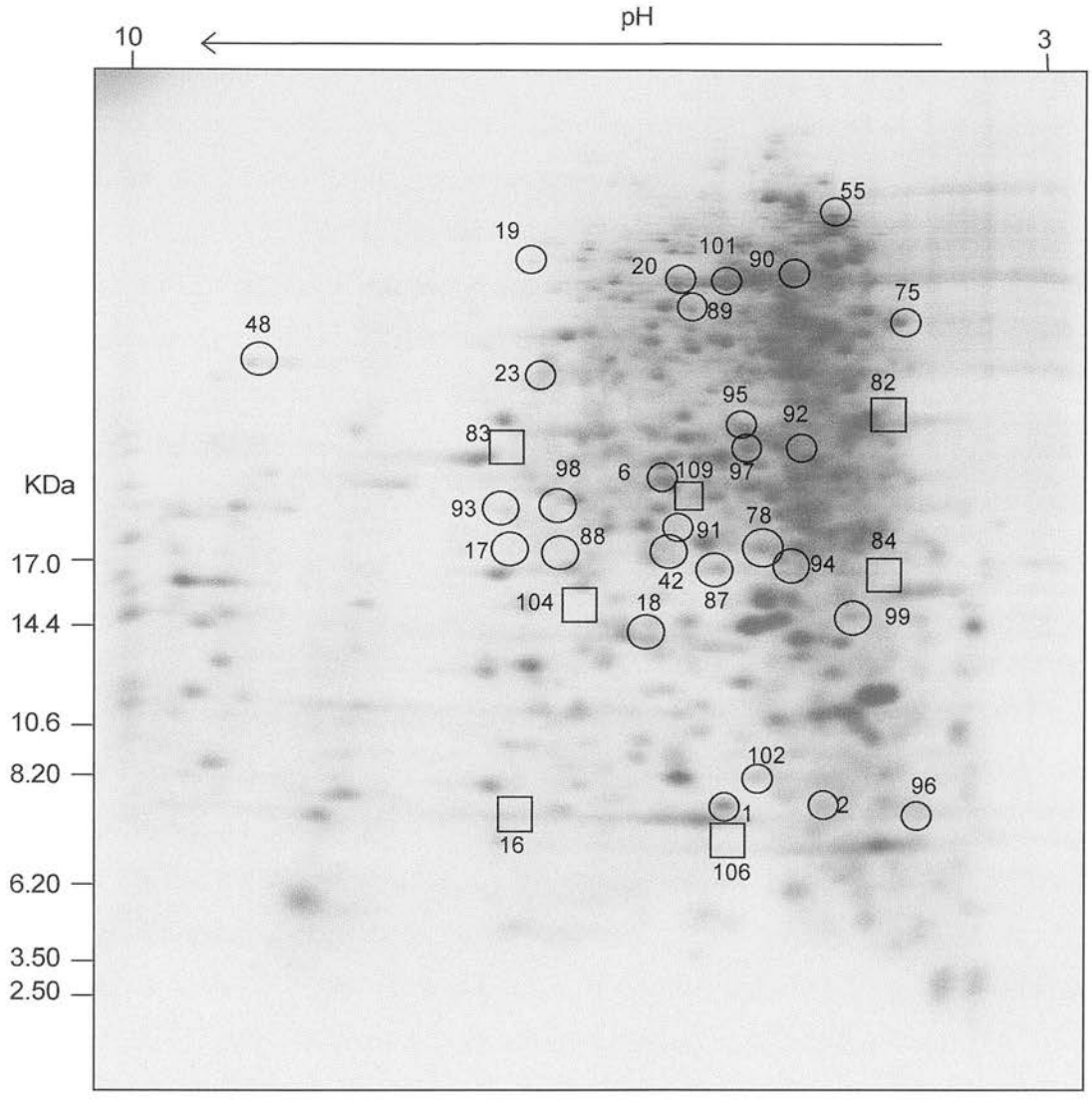


Figure 6.21 Autoradiograph of protein extracts from ^{35}S-methionine-labelled *S. typhimurium* MPG361 cells grown to mid-exponential phase at 30°C, in Spitizen medium. 3 ml of the culture was radio-actively labeled for 5 minutes at 30°C. The cells were disrupted by sonication and the protein extracts separated by 2-D PAGE as described in the legend for figure 6.1.

Protein spots whose synthesis was found subsequently to increase following a shift to low temperatures are circled. The positions of the boxes represent proteins that were only detected at low temperatures and were not evident at 37°C. The numbered proteins are listed in table 6.9.

Incubation of exponentially growing MPG361 cells at 10°C for 1.5 hours resulted in a dramatic decrease in overall protein synthesis, so that only 36 proteins above the threshold value were detected (figure 6.22). The level of synthesis of 7 proteins (1, 17, 18, 19, 20, 87 and 92) increased between 2 and 10-fold and a further 5 proteins (CspA (protein 1), 2, 42, 48, 75, 78) were induced more than 10-fold, relative to the level at 30°C. Proteins 1, 42 and 2 are shown in figure 6.23, panels A, B and C, respectively, as examples. CspA was the most abundant protein after 1.5 hours at 10°C and was found to have increased by 3.5-fold, relative to 30°C. 3 proteins above the threshold volume were detected that were not present at 30°C, proteins 82 (panel D, figure 6.23), 83 and 84. At this point 18 proteins were detected at a lower level relative to that at 30°C.

Extended incubation of exponentially growing MPG361 for 4 hours at 10°C resulted in an overall increase in *de novo* protein synthesis (figure 6.24). 93 proteins were detected above the threshold volume. Of these, 13 proteins (1, 17, 55, 91, 92, 93, 94, 95, 96, 97, 98, 99, 101) were induced between 2 and 10-fold and 10 (2, 19, 20, 42, 48, 75, 87, 88, 89, 90) were induced more than 10-fold relative to the level detected at 30°C. Expression of proteins CspA, 42 and 2 (figure 6.23, panels A, B and C, respectively) was observed at a maximum level after this extended incubation at 10°C. Proteins 109, 104 (panels A and B, respectively of figure 6.25) and 106 were not detected at 37°C and found to be most abundant after 4 hours at 10°C. 25 proteins were detected at a lower level after incubation for 4 hours at 10°C than at 30°C.

As many as 31 proteins were induced between 2 and 10-fold after 4 hours at 10°C and 19 were induced more than 10-fold, relative to the levels detected after 1.5 hours at 10°C. Only 8 proteins were detected at a lower level after incubation for 4 hours at 10°C, relative to their level after 1.5 hours.

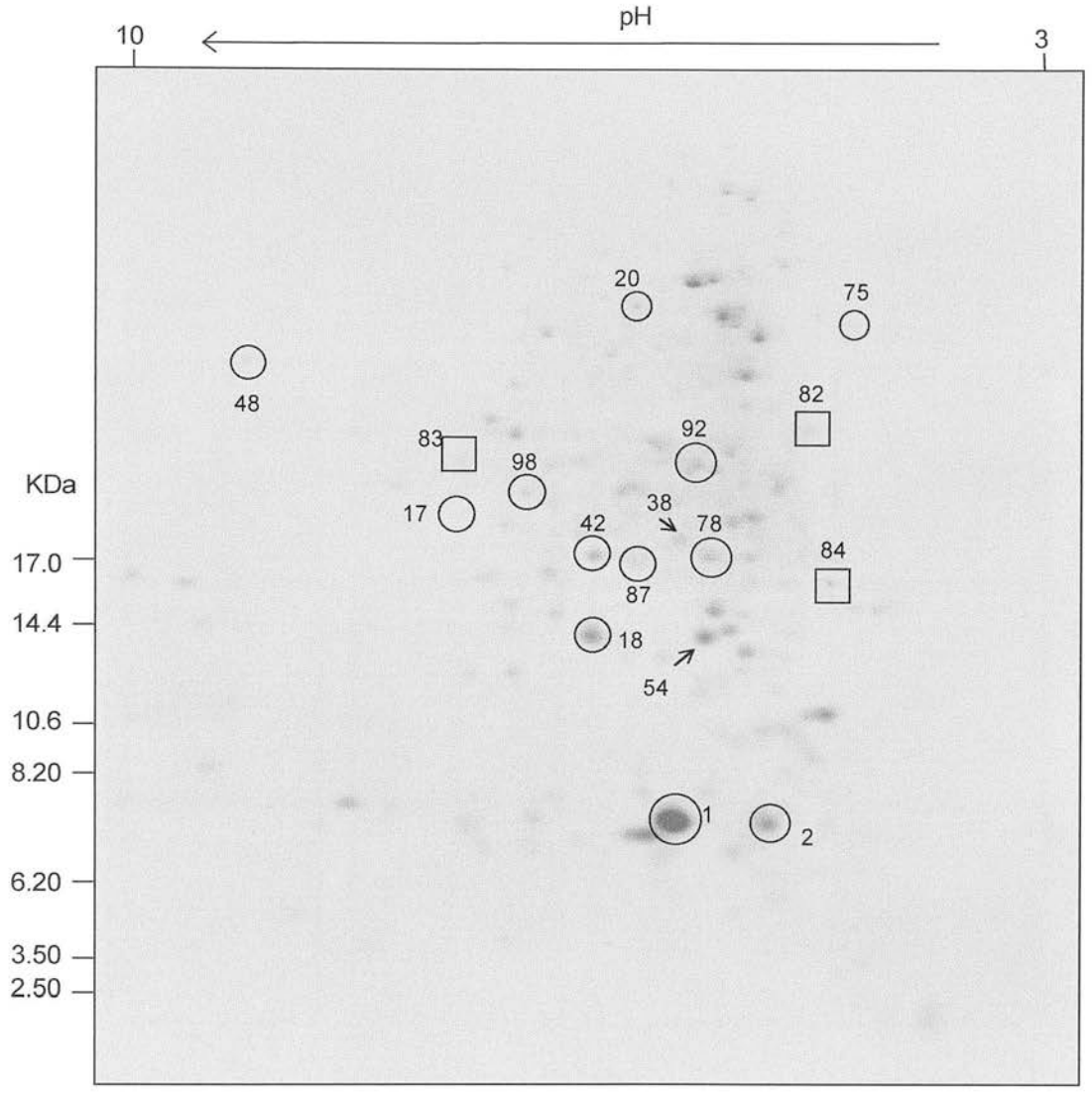


Figure 6.22 Autoradiograph of protein extracts from ^{35}S-methionine-labelled *S. typhimurium* MPG361 cells grown to mid-exponential phase at 30°C, in Spitizen medium, and incubated at 10°C for 1.5 hours. 3 ml of the culture was radio-actively labeled for 30 minutes at 10°C. The cells were disrupted by sonication and the protein extracts separated by 2-D PAGE as described in the legend for figure 6.1.

Protein spots whose synthesis was found subsequently to increase following a shift to low temperatures are circled. The positions of the boxes represent proteins that were only detected at low temperatures and were not evident at 37°C. The numbered proteins are listed in table 6.9. Other proteins of interest have been indicated with an arrow.

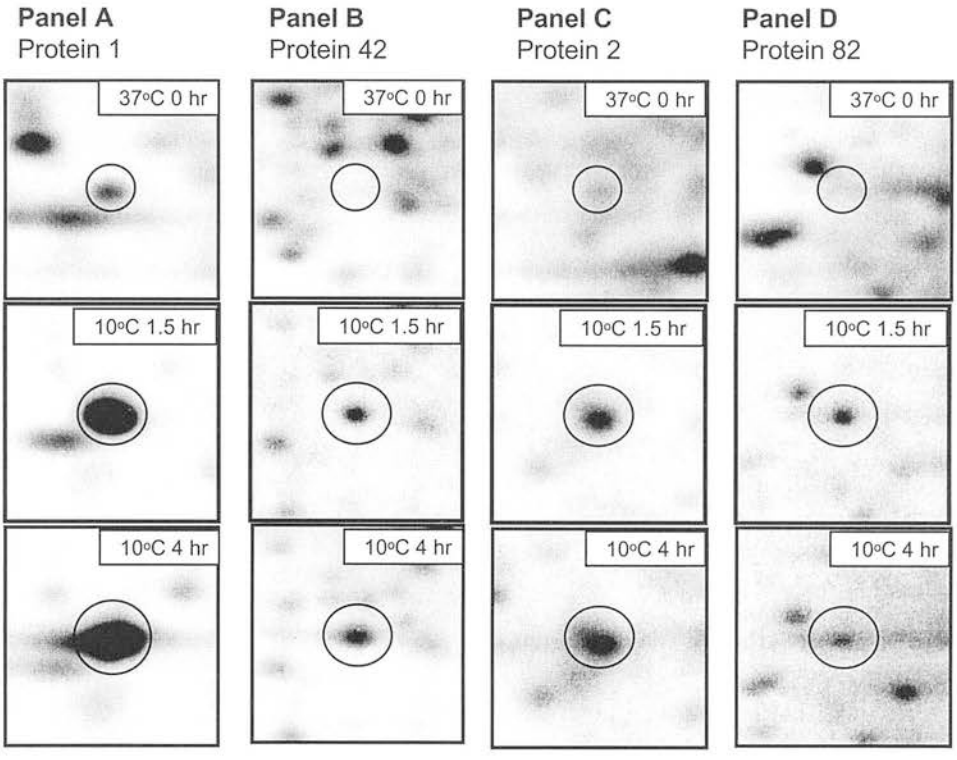


Figure 6.23

Montages showing cold shock induction of proteins from exponentially growing MPG361 cells, at 37°C and 10°C. Panel A, Protein 1; panel B, protein 42; panel C, protein 2; panel D, protein 82.

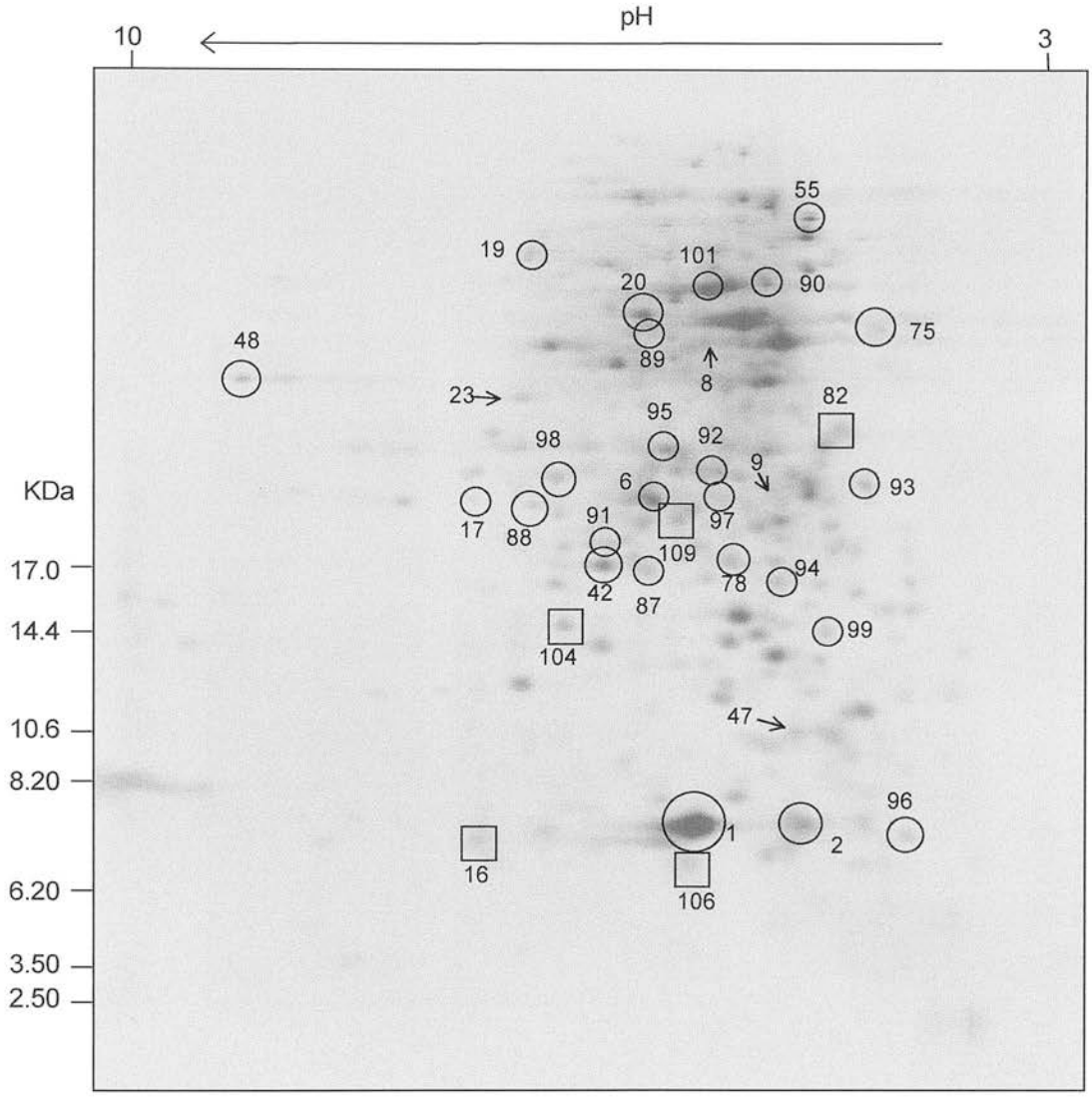


Figure 6.24 Autoradiograph of protein extracts from ^{35}S-methionine-labelled *S. typhimurium* MPG361 cells grown to mid-exponential phase at 30°C, in Spitizen medium, and incubated at 10°C for 4 hours. 3 ml of the culture was radio-actively labeled for 30 minutes at 10°C. The cells were disrupted by sonication and the protein extracts separated by 2-D PAGE. The cells were disrupted by sonication and the protein extracts separated by 2-D PAGE as described in the legend for figure 6.1.

Protein spots whose synthesis was found subsequently to increase following a shift to low temperatures are circled. The positions of the boxes represent proteins that were only detected at low temperatures and were not evident at 37°C. The numbered proteins are listed in table 6.9. Other proteins of interest have been indicated with an arrow.

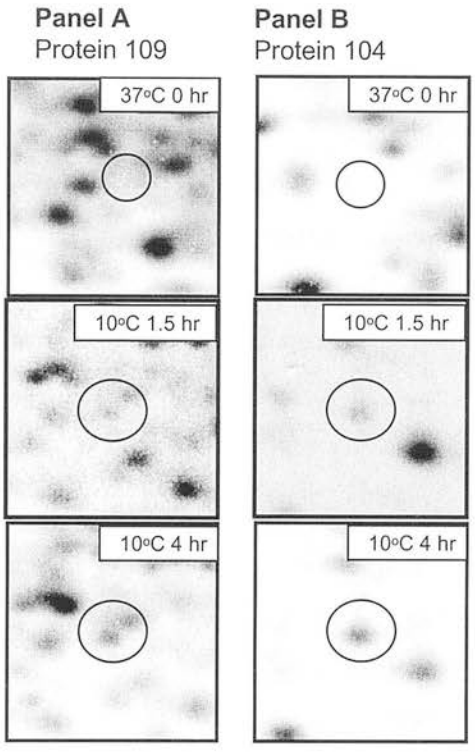


Figure 6.25

Montages showing cold acclimation proteins from exponentially growing MPG361 at 37°C and 10°C. Panel A, Protein 109; panel B, protein 104.

Table 6.9 list the proteins that were found to be cold shock inducible in exponentially growing MPG361 cells, at 10°C. Only 2 proteins (48 and 18) were found to be transiently induced at 10°C. In contrast, as many as 34 proteins were detected at a high level after incubation for 4 hours at 10°C, relative to the level detected at 30°C. 7 proteins (16, 82, 83, 104, 106, 109) that were cold-induced were not detected at 30°.

Protein number	pI	MW (KDa)	Incubation details 1.5 hours, 10°C	4 hours, 10°C
1	5.79	7.18	>	>
2	5.07	7.13	>>	>>
6	6.1	22.55	<	-
16	7.37	7.04	LTP	>
17	5.34	19.91	>	>
18	6.42	13.52	>	-
19	4.93	47.88	>	>>
20	5.64	45.31	>	>>
42	6.39	17.76	>>	>>
48	5.24	32.97	>>	>>
55	4.95	53.48	<	>
75	5.14	37.6	>>	>>
78	5.51	17.7	>>	>>
82	7	26.89	LTP	<
83	5.61	24.24	LTP	
84	4.58	16.24	LTP	nc
87	6.08	17.34	>	>>
88	4.83	19.7	<	>>
89	6.08	41.85	-	>>
90	5.23	45.78	<	>>
91	6.7	19.03	-	>
92	5.46	23.74	>	>
93	4.54	22.47	<	>
94	5.2	16.29	-	>
95	4.89	25.89	<	>
96	4.28	6.8	-	>
97	6.04	22.08	<	>
98	4.98	22.55	-	>
99	4.87	13.66	<	>
101	5.49	45.94	-	>
102	5.53	7.91	<	-
104	6.69	14.59	LTP	>
105	5.27	41.42	<	-
106	5.86	6.39	LTP	>
109	5.85	20.61	LTP	>

Table 6.9 Cold shock inducible proteins detected from cultures of MPG361 that were in exponential phase prior to temperature reduction from 30°C to 10°C. The proteins are highlighted in figures 6.21. 6.22, or 6.24. The apparent isoeletric point and molecular weights of the proteins were calculated from a set of 2-D standards, described in chapter 2 of this thesis. The relative levels of abundance have been given for the proteins detected after 1.5 and 4 hours of incubation at 10°C. The abbreviations and symbols are as described for table 6.5.

6.2.6 Identification of *S. typhimurium* CspA.

The 2-D PAGE results of samples from SL1344 cells had shown that a 7 KDa protein was highly induced following a decrease in temperature regardless of both the growth phase and the extent of the temperature decrease. The characteristics of this protein on 2-D PAGE indicated that it was likely to be a member of the CspA family and N-terminal sequencing, described below, showed that the protein was CspA.

The protein was isolated by 2-dimension gel electrophoresis. In order that the correct protein was isolated and N-terminal sequenced, whole cell extracts containing the protein were separated on parallel sets of 2-D gels. One set of samples was radioactively labelled, in the manner described previously. A second, parallel set of samples was unlabelled and proteins were extracted from a greater number of cells (the number of cells was increased 8-fold), which provided sufficient protein for N-terminal sequencing. The cultures were sampled at 37°C, prior to a temperature down-shift and at 10°C, 1.5 hours after the down-shift. Both sets of samples were processed at the same time, in the manner described above. The proteins were also resolved by 2-dimensional electrophoresis at the same time. Proteins from the labelled and unlabelled gels were transferred to PVDF membrane. The membrane containing the radioactively labelled proteins was embedded in the EA-Wax fluor and exposed to X-ray film for 24 hours, described in Holden *et al.* (1999). The PVDF membrane containing the unlabelled proteins was stained with amido black. The stained CspA spot was located by aligning the position of the proteins and markers with the autoradiograph. The CspA spot was excised from the PVDF membrane and N-terminal sequenced using the micro-Edman degradation technique, described by Hayes *et al.* (1989). The abundance of each amino acid residue (in pmol) for each cycle is shown (table 6.10). The resulting 10 amino acid sequence was found to be identical to the first residues 2 to 11 of *S. typhimurium* CspA and *E. coli* CspA (figure 6.26). Thus the cold-inducible protein was confirmed as *S. typhimurium* CspA. It should be noted that in the tertiary structure the initial methionine residue has been processed, which was also reported for *E. coli* CspA (Feng *et al.*, 1998).

A.A	Cycle number									
	1	2	3	4	5	6	7	8	9	10
Asp	nd	nd	nd	0.011	nd	nd	nd	nd	nd	nd
Asn	1.374	0.890	1.208	1.233	0.712	0.788	0.673	0.979	0.940	0.737
Ser	**58.32**	7.627	1.827	1.498	1.319	1.443	0.975	0.893	1.250	1.017
Thr	0.050	0.680	0.491	2.506	**25.93**	10.94	0.037	0.327	0.692	0.516
Gly	4.335	**47.91**	17.59	7.136	3.694	**24.68**	13.49	7.315	5.065	4.618
Glu	2.264	1.416	1.312	1.463	1.126	1.300	1.114	1.161	0.987	0.952
His	23.88	3.771	0.835	2.099	6.288	1.253	2.517	2.099	4.607	3.771
Ala	6.828	3.670	2.177	2.333	2.115	2.581	1.850	1.897	1.990	1.866
Arg	5.367	1.291	2.449	0.469	1.526	1.174	2.331	1.627	1.862	0.587
Tyr	0.669	0.357	0.507	0.473	0.346	0.496	0.357	0.426	0.173	**0.403**
Pro	1.942	1.282	1.262	1.521	1.081	1.425	1.588	1.37	0.995	1.310
Cys	77.34	46.95	27.61	16.57	24.85	38.67	30.38	27.61	46.95	44.19
Met	0.571	0.160	0.160	**5.63**	0.011	0.457	0.068	0.160	0.011	0.034
Val	1.563	1.356	1.304	1.705	1.808	2.118	2.622	**11.43**	6.550	3.320
Trp	0.987	1.380	0.350	nd	nd	0.435	nd	nd	0.636	2.760
Phe	1.472	1.711	0.970	1.450	nd	1.199	0.316	0.883	1.003	2.060
Ile	2.767	3.284	9.509	5.804	3.984	3.704	**33.71**	22.95	8.324	4.932
Leu	1.463	0.157	1.294	1.431	1.2	1.021	1.189	1.463	1.2	1.263
Lys	0.860	0.860	**12.83**	4.204	0.748	0.648	0.187	0.860	**3.867**	2.108

Table 6.10 Abundance of amino acid residues (pmol) for each of the first 10 cycles of the Edman degradation of CspA. The values in bold indicate the most abundant amino acid residue for each cycle. (nd = no data).

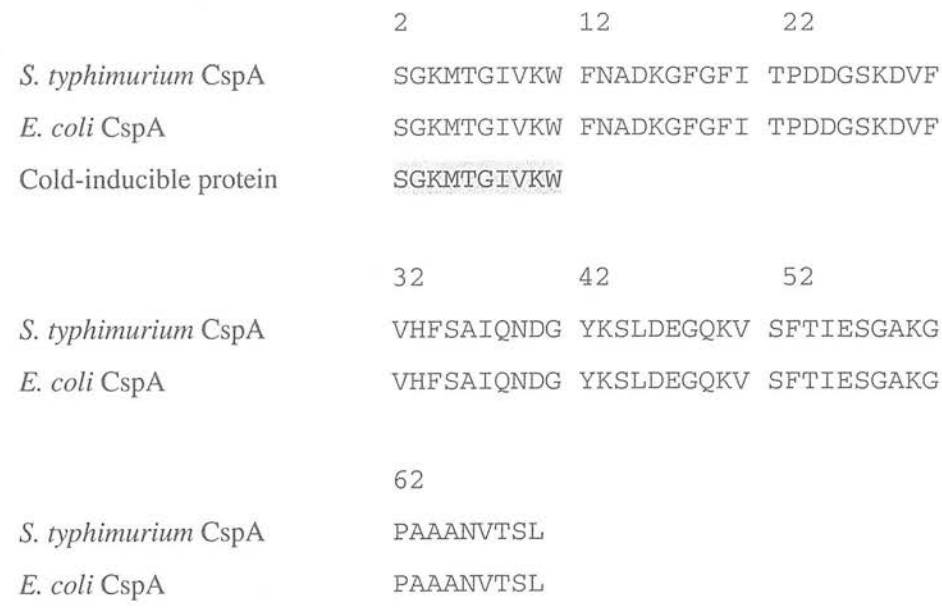


Figure 6.26 Alignment of the N-terminal sequence from the cold shock inducible protein with *S. typhimurium* CspA and *E. coli* CspA. The shaded area indicates the amino acid residues that were obtained from N-terminal sequencing.

The confirmation of the N-terminal amino acid sequence of *S. typhimurium* CspA allowed subsequent identification of the CspA protein spot on the 2-D PAGE gels, from both the position of the resolved protein on the gel and from the characteristic high level of induction. Thus, the CspA protein spot was one of the spots used for pI and molecular weight calibration for the 2-D gels.

6.3 Discussion

Frequently, bacterial stress responses have been characterised by analysing the proteome under different conditions. Studying changes in expression of proteins provides an opportunity to uncover the dynamics of a stress response. Individual proteins that are most affected by the stress are highlighted and subsequently can be further characterised.

The cold shock response of mesophilic bacteria has been studied most extensively in *E. coli* and *B. subtilis*, for cultures that are in exponential phase at the point of the temperature reduction. In the cold shock response of *E. coli* an initial lag in growth occurs that lasts for approximately 4 hours. Interestingly the lag period was observed only when the cells were grown in defined MOPS media (Jones *et al.*, 1987; Jones *et al.*, 1992), whereas no lag was observed when the cells were grown in rich broth (Broeze *et al.*, 1978). During this time, overall protein synthesis decreased together with induction of a specific set of cold shock proteins (Jones *et al.*, 1987). After approximately 4 hours at 10°C, overall protein synthesis increased to levels that were similar to that observed prior to the temperature decrease, although, some cold induced proteins were still synthesised at a higher level that that observed at 37°C (Jones *et al.*, 1987).

Cold-induced proteins have been detected in many bacteria since the initial findings in exponentially growing *E. coli* by Jones *et al.* (1987). In an exponentially growing culture of *E. coli* that had been shifted from 37°C to 10°C, 13 proteins were induced, detected by 2-D PAGE. An earlier study on the effect of temperature on protein synthesis had also shown that 22 proteins were induced at least 2-fold in a culture of exponentially growing *E. coli* (Herendeen *et al.*, 1979). To date, 4 members of the *E. coli* CspA family are known to be induced following a temperature decrease (Lee *et al.*, 1994; Nakashima *et al.*, 1996, Wang *et al.*, 1999).

The cold shock response of *B. subtilis* is similar to that observed in *E. coli* in terms of the dynamics of overall protein synthesis (Graumann *et al.*, 1996). However, no lag period in growth was observed when a culture of exponentially growing *B. subtilis*

was shifted from 37°C to 15°C. In this case, the cells were grown in minimal M9 medium. Shifting the culture to 15°C resulted in induction of a total of 38 proteins over a period of 2 hours. Included in this set of proteins was 3 members of the CspA family, all of which were induced to a high level after 30 minutes of incubation at 15°C (Graumann *et al.*, 1996). 6 of the proteins that were cold-induced were also induced in response to heat shock (48°C) and 7 were also induced in response to osmotic shock (1 M NaCl) (Graumann *et al.*, 1996). One protein was induced following exposure to all 3 stress conditions and micro-sequence analysis showed that it was involved in the phosphoenolpyruvate:sugar phosphotransferase system.

The study in this chapter has shown that *S. typhimurium* SL1344 exhibits a dynamic cold shock response similar to that seen in other mesophilic bacteria such as *E. coli* and *B. subtilis*. Shifting an exponentially growing culture of *S. typhimurium* from 37°C to 10°C resulted in an initial decrease in overall protein synthesis together with transient induction of a set of 13 proteins (termed CIPs). After prolonged incubation at 10°C, overall protein synthesis increase to levels similar to that observed prior to the downshift. 15 proteins (termed CAPs) were most highly induced at this point. Furthermore, 7 proteins were expressed at 10°C that were not detected at 37°C. Comparison of the cold-induced proteins in *S. typhimurium* with the known cold-induced *E. coli* proteins lead to the provisional identification of SdhA (4), Hsc66 (55), SucB (20), CarA (8), OmpA (43), SodA (6), IbpB (40), RbfA (18) and H-NS (54) (Jones *et al.*, 1987; VanBogelen *et al.*, 1996). The numbers in parenthesis refer to the *S. typhimurium* proteins identified by 2-D PAGE (figures 6.1, 6.2, 6.4 and 6.13). It should be noted that these identifications are based on comparison of apparent isoeletric points and molecular sizes of the *S. typhimurium* proteins with the actual physical parameters of the *E. coli* proteins. Thus, the protein identifications are theoretical. Attempts to identify the amino acid sequence of some of the *S. typhimurium* cold inducible proteins by nano-spray mass spectrometry unfortunately proved unsuccessful.

The major cold shock protein was identified by N-terminal sequencing. 10 amino acid residues were identified from Edman degradation and shown to be identical to

the residues 2 to 11 of the putative peptide encoded by *S. typhimurium cspA* (Craig & Gallagher, unpublished data) and *E. coli* CspA (Goldstein *et al.*, 1990). CspA was found to be induced 200-fold following incubation for 1.5 hours at 10°C and the level decreased after incubation for 4 hours at 10°C, although it was approximately 19-fold higher than that observed prior to the downshift.

Storage of foodstuffs at low temperature is a widely used measure to minimize the number of associated bacteria. However, some pathogens, such as *L. monocytogenes* are able to grow at such temperatures, and it has been suggested that refrigeration at 4°C therefore acts as effective enrichment for these organisms (Farber & Peterkin, 1991). The minimum growth temperature for *S. typhimurium* is approximately 7°C (Mossel *et al.*, 1981), which is above the temperatures used refrigeration. However, there are very few reports of protein synthesis of salmonellae at below the minimum growth temperatures (for a recent report see Jeffreys *et al.*, 1998). Thus, protein expression of *S. typhimurium* SL1344 was investigated following a temperature downshift from 37°C to 4°C, and subsequent incubation at 4°C for 96 hours. In general, the response at this temperature was similar to that observed at 10°C, although, important differences occurred. The adaptation period was extended by several hours and it appeared that the acclimation phase occurred after 24 hours of incubation at 4°C. In contrast, the acclimation phase occurred after only 4 hours of incubation at 10°C. Furthermore, after incubation of 96 hours at 4°C, the number of newly synthesised proteins detected was only about 40 % of that detected after 4 hours at 10°C. At 4°C, CspA was detected at a maximum observed level after 2 hours of incubation and was induced 134-fold relative to the level detected prior to the temperature reduction. At subsequent time points, the level of CspA decreased and was induced approximately 10-fold higher than that observed at 37°C, following 96 hours of incubation at 4°C. Following prolonged incubation, at least 3 cold-induced proteins (34, 50, 57) were detected at a higher level at 4°C than at 10°C. In addition, 1 protein (33) that was not detected at 37°C, was expressed at a maximum level after 96 hours at 4°C.

This study unambiguously shows *de novo* protein synthesis at a temperature at which multiplication of *S. typhimurium* does not occur. In an earlier study with *E. coli*, Broeze and colleagues (1978) showed that incubation of an exponentially growing culture of *E. coli* at 5°C led to a block in translation. This data supports the finding that overall protein synthesis in *S. typhimurium* decreases when shifted to either 10°C or 4°C. However, the study by Broeze and colleagues (1979) investigated protein synthesis in *E. coli* for a maximum of 60 minutes after downshift to 5°C. It appears that *S. typhimurium* overcomes the block in protein synthesis after prolonged incubation, both above and below the minimum temperature permissible for growth.

De novo protein synthesis has also been investigated from an exponentially growing culture of *S. typhimurium* that contained a mutated version of a second cold inducible member of the CspA family, CspB. The dynamics of the cold shock response at 10°C was similar to that for the wild type strain, although, some differences were observed. It appeared that CspA was detected at 30°C (figure 6.22) and the level of CspA was higher in the *cspB*::Mu*dlux* strain after prolonged incubation at 10°C relative to the wild type strain(figures 6.4 and 6.24). However, it is important to note that such an observation is inconsistent with the body of work published on the thermo-regulation of *E. coli* CspA. If *S. typhimurium* CspB was somehow able to down-regulate CspA at 30°C, it would require a hitherto unknown method of regulation. This is unlikely since it is known that *cspB* is cold-shock inducible and is not detectable at 30°C (chapter 4 of this thesis). Furthermore, the identity of this protein in cultures of MPG361 was not confirmed by sequencing, although the molecular size and isoelectric point suggested that it was very similar to CspA. However, it is likely that another protein with very similar pI and molecular size to CspA has been detected on the autoradiograph in a very similar position to the one occupied by CspA. The presence of such a protein would alter the actual protein spot volume calculated for CspA, therefore, the protein spot volume should be viewed with a degree of caution.

An alternative possibility is that *S. typhimurium* CspA is expressed at 30°C, but not at 37°C. 2-D PAGE analysis of *S. typhimurium* SL1344 (chapter 6 of this thesis) and

reporter studies of a transcriptional fusion to the *S. typhimurium cspA* promoter show that production of β-galactosidase was low 37°C (chapter 4 of this thesis). Similarly *E. coli* CspA was not found to be expressed at this temperature, although thermo-regulation studies have shown that this protein was induced when an exponentially growing culture was shifted from 37°C to 30°C, albeit to a low level (Etchegaray *et al.*, 1996). Recent evidence suggests that *E. coli* CspA is highly induced following during the lag phase, at 37°C, although when the cells enter exponential phase, the level of CspA decreases to a minimum (Brandi *et al.*, 1999). Further evidence of 'high temperature' expression of major cold shock proteins has been shown with *B. subtilis*. 2D-PAGE studies indicated that *B. subtilis* CspB was expressed at low levels at 37°C. In this case, exponentially growing *B. subtilis* cells were labelled with ^{35}S-methionine for 10 minutes at 37°C. Interestingly, although CspB protein was evident at 37°C in this report, previous gene reporter studies indicated that expression was minimal at 37°C, when *B. subtilis* cells were exponentially growing (Willimsky *et al.*, 1992). Taken together, these studies suggest that the major cold shock protein may be transiently stabilised at 30°C.

The level of *S. typhimurium* CspA appeared to be higher in exponentially growing MPG361 cells after 4 hours of incubation at 10°C than observed in wild type cells. In contrast, the maximum level of CspA from wild type cells was observed after 1.5 hours incubation at 10°C. Previous studies of the effects of mutation of the major cold shock proteins of *E. coli* (Bae *et al.*, 1997) and *B. subtilis* (Graumann *et al.*, 1997) on overall protein expression at low temperatures, have shown that expression of further CspA homologues was affected by the mutation. In *E. coli* synthesis of both CspB and CspG was shown to increase in a Δ*cspA* strain relative to the wild type strain (by approximately 1.3-fold). In addition, their expression was prolonged for at least 3 hours, relative to that observed the wild type strain (Bae *et al.*, 1997). However, neither CspB nor CspG reached the same level of induction as observed for CspA, when *cspA* was disrupted. Disruption of *B. subtilis cspB* appeared to affect the expression of several proteins, including CspC and CspD, when exponentially growing cells were shifted from 37°C to 15°C. The synthesis of these proteins increased relative to the wild type cells (Graumann *et al.* 1996). Thus it appears that

cold inducible members of the CspA family may compensate for the loss of another member. It would seem likely, therefore, that a similar effect may occur in *S. typhimurium*, especially after prolonged incubation at 10°C.

Several of the proteins detected in exponentially growing SL1344 cells and MPG361 cells seemed to be concurrent, judging by their apparent pI and molecular sizes. In both strains proteins 4, 9, 38 and 47 were most highly induced after 4 hours at 10°C. In both strains protein 2 was induced at a maximum level after prolonged incubation at 10°C, and was not previously detected at 37°C. Proteins 6, 8, 23 and 1 (CspA) were most highly induced after 1.5 hours at 10°C in exponential phase cultures of SL1344. In contrast, the level of synthesis of these proteins was highest after 4 hours at 10°C in exponential phase cultures of MPG361. The delayed induction of some of these proteins may be linked to the increase in the level of the putative CspA protein, after prolonged incubation at 10°C. In wild type cells, the maximal level of induction of these proteins was found to coincide with that of CspA. From the studies in this chapter it is clear that mutation of *S. typhimurium cspB* led to significant differences in the cold shock response.

It is well known that non-differentiating cells, such as *Salmonella*, that are in stationary phase are very resistant to environmental challenges (Kolter *et al.*, 1993). Furthermore, pathogens, such as *Salmonella*, that are sampled from the 'field', e.g. from processed animal carcasses, are more likely to be in stationary phase when they are sampled, since the conditions are unlikely to support optimal growth. Thus, in order to determine the effect on protein expression under similar conditions, the cold shock response of *S. typhimurium* was investigated when the cells were in stationary phase of culture.

Shifting a stationary phase culture of *S. typhimurium* from 37°C to 10°C resulted in a decrease in overall protein synthesis, together with induction of 5 proteins (58, 59, 60, 63, 64). Only 2 proteins (1, 2) were induced above the threshold level at 10°C that were not detected at 37°C, one of which was CspA (deduced from the level of expression and the apparent isoelectric point and molecular size). Extended

incubation at 10°C led to further reduction in overall protein synthesis, which contrasts to the situation observed when the cells were in exponential phase. Thus, the dynamics of the cold shock response of *S. typhimurium* differ in a growth-phase dependent manner.

The cold shock response of stationary phase *S. typhimurium* at 4°C was similar to that observed at 10°C. However, 1 protein (64) was induced to a slightly higher level at 4°C, relative to that observed at 10°C. Interestingly, the only protein that was detected after prolonged incubation at 4°C was CspA.

It was clear that the kinetics of expression of CspA differed with the change in growth phase. Although the level of induction of CspA was similar in exponential phase cultures and stationary phase cultures that had been incubated for 1.5 hours at 10°C, a higher level of CspA was detected in stationary phase cultures after 4 hours at 10°C. Furthermore, in general, *de novo* protein synthesis decreased after prolonged incubation at 10°C in stationary phase cultures. In contrast, in exponential cultures the level of CspA decreased after 4 hours at 10°C, which coincided with an increase in overall protein synthesis. Overall, there did not seem to be a great deal of similarity between the proteins induced in exponentially growing cultures and stationary phase cultures. However, induction of CspA (and other proteins, such as protein 2) were common to both exponential and stationary phase cultures. Protein 15 was also evident in stationary phase cultures for at least 4 hours at 10°C and 12 hours at 4°C. This suggests that CspA, and possibly further cold-induced proteins, play a major role in adaptation to low temperatures, in a growth phase-independent manner.

The use of 2-D PAGE has been fundamental for the characterisation of many bacteria stress responses. However, the results should be interpreted with inherent limitations in mind. The proteins identified in the study of the effects of low temperature on protein synthesis in *E. coli* (Herendeen *et al.*, 1979) were radioactively labelled with ^{14}C-leucine whereas those identified in the study by Jones and colleagues (1987) were labelled with ^{35}S-methionine. This may have lead to

differences in the proteins detected. However, such differences are likely to be very small since very few proteins lack either leucine or methionine. An important consideration is the use of various pH gradients and molecular weight ranges to separate proteins. There is a trade-off between the level of resolution the range of protein detection. For example, the use of a wide pH range (from pH 3 to 10) in the 1st dimension of a study on stress-induced proteins from *B. subtilis*, led to the successful identification of members of the CspA family with relatively acidic isoelectric points, around pH 4.3 (Graumann *et al.*, 1986). Limitations in the 2nd dimension may also lead to some proteins being missed. In particular, members CspA family of proteins, which appear to be a fundamental part of the cold shock response, have a small molecular size, around 7 KDa, and therefore may be poorly resolved on gels with a molecular size range does not extend beyond this size. The study in this chapter has resolved proteins using a wide pH gradient in the 1st dimension and a low molecular weight system for the 2nd dimension, to maximize the number of proteins detected with a small molecular weight.

A further limitation arises from the reliance of computerised analysis software. On one hand this technique undoubtedly detects a greater number of proteins that can be easily detected by eye. However, comparison of gels that are markedly different require a substantial level of manual protein matching. Automatic protein matching from one gel to another frequently resulted in obviously erroneous protein matches, which had to be altered manually. Thus, the more disparate the protein gels, the more likely that errors would arise during protein matching. These limitations should be borne in mind during interpretation of the 2-D PAGE analysis of the cold shock response of *S. typhimurium*.

In summary, *S. typhimurium* was shown to exhibit dynamic and dramatic changes in protein synthesis following decreases in temperature which are dependent on the degree of the temperature reduction and the growth phase. CspA was the most abundant protein at both 10°C and 4°C, and in both exponential and stationary phase cultures. Mutation of CspB, which is also highly induced in the cold in *S. typhimurium* (Craig *et al.*, 1998, chapter 4 of this thesis), appeared to affect synthesis

of CspA prior to the temperature downshift and after extended incubation at 10°C. The level of induction of 28 proteins increased at least 2-fold when exponentially growing *S. typhimurium* cells shifted from 37°C to 10°C, whereas, only 5 proteins were induced between 2 and 10-fold in stationary phase cultures. Mutation of CspB did not affect the numbers of cold shock inducible proteins in exponentially growing cultures, although peak level of induction of many of these proteins was delayed at 10°C. This study shows that *S. typhimurium* responds to low temperature in a similar way to other mesophilic bacteria. Many authors believe that the cold shock response is an adaptive mechanism that enables bacteria to survive at low temperatures. From this study it is clear that *S. typhimurium* is also able to adapt to low temperatures.

We thank the Welmet Protein Characterisation Facility, University of Edinburgh for sequencing *S. typhimurium* CspA. Welmet is supported by the Wellcome Trust, Edinburgh and Heriot-Watt universities and the Salvesen's Trust.

CHAPTER 7

CONCLUDING REMARKS

Understanding the molecular basis of the cold shock response may provide valuable information that can be used to reduce the numbers of salmonellae associated with food, and thus reduce the risk of salmonellosis. In comparison to the body of work for other mesophiles, there are few reports of the cold shock response in *Salmonella* (Craig *et al.*, 1998; Jeffreys *et al.*, 1998). This response most probably plays an essential role in survival at low temperature (Graumann *et al.*, 1997) and therefore in the longer term, for the incidence of food-borne disease. Thus, it is important that the response be better characterised. Accordingly, this thesis has investigated aspects of the cold shock response of *S. typhimurium*.

7.1 Cold Shock response of *Salmonella typhimurium*

7.1.1 Disruption of the major cold shock gene of *S. typhimurium*

For this thesis, mutation of the gene encoding the major cold shock protein of *S. typhimurium* was attempted, in order to assess the role of CspA at low temperatures. This procedure required several stages of plasmid construction before allelic exchange was attempted. The *cspA* gene was disrupted by insertion of the *lacZ* reporter gene at position + 110 of *cspA* (relative to the transcriptional start site), which was 44 bp upstream of the translation start site. The entire untranslated region and approximately 160 bases preceding the transcriptional start site were included in the *cspA* DNA. Subsequent to addition of *lacZ*, the *cml* cassette was inserted immediately downstream of *lacZ*, to confer antibiotic resistance and a means of selection. This construct contained the promoter region from *cspA* and the ribosome binding site from *lacZ*. Recombination attempts with this construct were made in *S. typhimurium* strain SL1344, but did not result in recovery of recombinants. Thus, recombination was attempted in a *S. typhimurium* strain (CH607) that lacked a functional *polA* gene. It was thought that such a strategy would improve the possibility of recombination events since the *polA* gene product, DNA polymerase I, is required for replication of ColE1-based plasmids. A small number of colonies were recovered that were resistant to chloramphenicol and sensitive to ampicillin

(the *bla* gene was carried by the plasmid vector). Analysis of the DNA of these colonies indicated that a single cross-over recombination event had occurred with the DNA downstream of the *cspA* coding region (figure 3.8). Since a functional copy of *cspA* remained present on the chromosome in addition to the disrupted copy after such an event, these strains could not be used to assess the role of CspA at low temperatures.

Further attempts were made to recombine disrupted *cspA* DNA onto the chromosome using alternative strategies that may have improved the possibility of allelic exchange. It was proposed that the *lacZ-cml* DNA that replaced the *cspA* ORF was too long (figure 3.5A), which would physically reduced the possibility of allelic exchange. Thus, *cspA* was disrupted in a manner similar to the one described above, with the absence of *lacZ* as a reporter gene. In its place a promoterless *cat* gene was used which was expected to confer antibiotic resistance driven by the *cspA* promoter. At the same time, this construct had the ability to be used as a reporter system for *cspA* expression, by direct measurement of ^{14}C-labelled acetyl chloramphenicol (Shaw, 1975). When recombination attempts at 37°C did not result in recombinants, it was proposed that it was due to the unstable nature of *cspA* mRNA at 37°C. Studies with *E. coli* have shown that *cspA* mRNA is degraded within 10 seconds at 37°C (Goldenberg *et al.*, 1996), but is increasingly stable below 30°C, reaching a maximum half life at 15°C (Etchegaray *et al.*, 1996). Thus, recovery of potential recombinants was carried out at a temperature (23°C) at which *E. coli cspA* mRNA has been reported to be stabilised (Goldenberg *et al.*, 1996). However, no recombinants were recovered at this temperature.

The subsequent strategy involved truncating the DNA downstream of the *cspA* ORF, so that the flanking DNA that was homologous to the *cspA* locus was approximately the same length (figure 3.9A). The *cspA* ORF was replaced with a consituative *cml* cassette, which meant that this construct could not be used for reporter studies, but would still provide a useful tool for studying the role of CspA at low temperatures.

There was a possibility that the low recombination frequency observed in disruption attempts, was due to insufficient *cspA-cml* DNA for allelic exchange. Thus, linear DNA that was amplified by PCR, was used for recombination. In this form, the disrupted DNA was present in a 10-fold higher concentration than the re-circularised DNA that had been used previously. The host strain that was used for this recombination attempt was MPG490 (*recD::*Tn10)(Tc^R), which contained a *recD* mutation and has been reported to tolerate linear DNA (Russel *et al.*, 1989), thereby increasing the likelihood of recombination. This strategy yielded a small number of transformants that were resistant to chloramphenicol. However, DNA analysis of these strains revealed that the parental plasmid, which had been used as the template for PCR amplification of the linear disrupted DNA, had inadvertently been introduced into the host strain (figure 3.16). This occurred despite the very low concentration of plasmid that was carried-over in the PCR DNA purification.

Further attempts to mutate *S. typhimurium cspA* were made based on co-transduction of a selectable marker in close proximity to the *cspA* locus. (This was originally developed for construction of mutations in *E. coli* by McLennan and Masters (unpublished data)). This strategy involved sub-cloning the disrupted *cspA-cml* DNA into a high copy number plasmid, which was subsequently transformed into a strain that contained a genetic marker in close proximity to the *cspA* locus. P22 transduction was used to transfer the region encompassing the genetic marker and disrupted *cspA* DNA, to a wild type background. This strategy yielded only 1 potential recombinant. However, DNA analysis showed the presence of the high copy-number plasmid that contained the disrupted *cspA* DNA (figure 3.18). Furthermore, the plasmid had been truncated by approximately 1 Kbp and had lost resistance to ampicillin during the recombination attempt.

The difficulty in trying to mutate *S. typhimurium cspA* suggest that it may be an essential gene. However, this is unexpected since disruption of *E. coli cspA* has been carried out successfully, and without any reported deleterious effects to cell growth at 37°C or 15°C (Bae *et al.*, 1997). However, 2D PAGE analysis of soluble proteins from the Δ*cspA* strain following incubation for 3 hours at 15°C revealed that

synthesis of CspB and CspG increased relative to that observed in the wild type strain. This aberrant increase was transient, since further 2D PAGE analysis following 16 hours of incubation at 15°C showed that the protein profile was essentially the same as that of the wild type strain. It should be noted, however, that a positive and negative selection method was used for disruption of *E. coli cspA* (Bae *et al.*, 1997).

The most compelling evidence for an essential nature of CspA homologues comes from studies with *B. subtilis*. Initial studies showed that disruption of CspB, the major cold shock protein of *B. subtilis*, led to a reduction in survival following freezing stress (Willimsky *et al.*, 1992). Further studies showed that simultaneous mutation of CspB, CspC and CspD (CspA homologues that are cold shock-inducible) proved lethal to the cells at optimum temperatures. Viability was restored by complementation of *cspB*, on a plasmid (Graumann *et al.*, 1997). 2D PAGE analysis with the *B. subtilis* strain that lacked CspB, indicated that the major cold shock protein had a pleiotrophic effect on protein expression at 15°C (Willimsky *et al.*, 1992).

A possible explanation for the inability to mutate *S. typhimurium cspA* was that recombination was not attempted to saturation. Perhaps an increase in the efficiency of recombination would have led to a successful double recombination event. It is also possible that an alternative method for allelic exchange may have proved successful. For example, there are several methods based on the inability of temperature-sensitive plasmids to replicate at non-permissive temperatures, such as a method for allelic exchange in *E. coli* which utilises the pSC101 replicon (Hamilton *et al.*, 1989). Nevertheless, it is intriguing that mutation of *S. typhimurium cspA* eluded us despite the numerous strategies employed.

7.1.2 Expression and regulation of *S. typhimurium cspA* homologues.

Regulation of the *cspA*-like genes appears to be complex and involves several mechanisms at the level of DNA expression and transcription, mRNA stability and translation. An important route of regulation of these CspA homologues seems to be via stabilisation of the mRNA transcripts at low temperatures. Studies with *E. coli cspA* have shown that the transcript is extremely unstable at optimal temperatures, i.e. 37°C, but is substantially stabilised following incubation of bacteria at low temperatures (Fang *et al.*, 1997). It seems that RNaseE is partially responsible for the rapid degradation of the transcript at optimum temperatures, since mutation in the putative RNaseE site near the ribosome binding site in *E. coli cspA* resulted in a very large increase in mRNA stability at 37°C, relative to the wild type (Fang *et al.*, 1997).

Stabilisation of mRNA of *E. coli cspA* and *cspB* has been shown to be differentially regulated (Etchegaray *et al.*, 1996), while *S. typhimurium cspB* mRNA has been shown to be stabilised below threshold temperature of approximately 22°C, suggesting that a temperature dependent threshold switch exists for activation of *cspB* induction (Craig *et al.*, 1998). These data indicate that for some of the cold-induced CspA-like proteins, a high level of sophistication exists that enables the organism to detect small fluctuations in temperature.

Translation of the *E. coli cspA* mRNA transcript seems to be positively regulated by the presence of a putative downstream box (DB), located within the initial 10 codons of *cspA* (Mitta *et al.*, 1997). This element is, in part, complementary to the 16S rRNA anti-DB, which has been reported as playing a similar role as the ribosome binding sequence in enhancing mRNA association with ribosomes (Sprengart *et al.*, 1996). On the other hand, an 11 bp region located immediately downstream of the transcriptional start site of *E. coli cspA*, *cspB* and *csdA*, appears to be necessary for negative regulation of the corresponding cold shock genes (Jiang *et al.*, 1996). It has been proposed that the cold box is a repressor binding site and further studies with *E.*

7.1.2 Expression and regulation of *S. typhimurium cspA* homologues.

Regulation of the *cspA*-like genes appears to br complex and involves several mechanisms at the level of DNA expression and transcription, mRNA stability and translation. An important route of regulation of these CspA homologues seems to be via stabilisation of the mRNA transcripts at low temperatures. Studies with *E. coli cspA* have shown that the transcript is extremely unstable at optimal temperatures, i.e. 37°C, but is substantially stabilised following incubation of bacteria at low temperatures (Fang *et al.*, 1997). It seems that RNaseE is partially responsible for the rapid degradation of the transcript at optimum temperatures, since mutation in the putative RNaseE site near the ribosome binding site in *E. coli cspA* resulted in a very large increase in mRNA stability at 37°C, relative to the wild type (Fang *et al.*, 1997).

Stabilisation of mRNA of *E. coli cspA* and *cspB* has been shown to be differentially regulated (Etchegaray *et al.*, 1996), while *S. typhimurium cspB* mRNA has been shown to be stabilised below threshold temperature of approximately 22°C, suggesting that a temperature dependent threshold switch exists for activation of *cspB* induction (Craig *et al.*, 1998). These data indicate that for some of the cold-induced CspA-like proteins, a high level of sophistication exists that enables the organism to detect small fluctuations in temperature.

Translation of the *E. coli cspA* mRNA transcript seems to be positively regulated by the presence of a putative downstream box (DB), located within the initial 10 codons of *cspA* (Mitta *et al.*, 1997). This element is, in part, complementary to the 16S rRNA anti-DB, which has been reported as playing a similar role as the ribosome binding sequence in enhancing mRNA association with ribosomes (Sprengart *et al.*, 1996). On the other hand, an 11 bp region located immediately downstream of the transcriptional start site of *E. coli cspA*, *cspB* and *csdA*, appears to be necessary for negative regulation of the corresponding cold shock genes (Jiang *et al.*, 1996). It has been proposed that the cold box is a repressor binding site and further studies with *E.*

coli cspA suggest that CspA (Bae *et al.*, 1997), and more recently, CspE (Bae *et al.*, 1999), are the repressors that bind to the cold box or mediate this repression.

Recent work by Bae and colleagues (1999) has shown that *E. coli* CspE, which is not cold shock inducible, appears to regulate expression of *cspA* at the level of transcript elongation or transcript termination, but not at the level of transcript initiation. Moreover, in a *cspE* null mutant strain, CspA protein was synthesised at 37°C, in exponentially growing cells. The level of CspA was approximately 40 % of that observed from cells that had been incubated at 15°C for 2 hours. Further *in vitro* investigation has indicated that CspE may increase RNA polymerase pause recognition of the *cspA* transcript, specifically at the 'cold box' region (Bae *et al.*, 1999). *In vitro* transcription experiments using RNA polymerase bound to an immobilised matrix and incubated with a 310 bp template that contained the *cspA* promoter and 5'-UTR, suggested that RNA polymerase pause recognition increased approximately 2-fold immediately downstream of the cold box, in the presence of purified CspE. Addition of purified CspA led to similar findings. Interestingly, CspE did not appear to affect expression of other cold shock genes that also contain a cold box region.

For this thesis gene expression of *S. typhimurium cspA* was examined using a transcriptional *lacZ* reporter system. A *cspA-lacZ* construct was made, on a plasmid, and transformed into a *pcnB E. coli* host that maintained the plasmid at a low copy number (Lopilato *et al.*, 1986). This construct included several of the putative regulatory elements reported previously for the *E. coli cspA* locus, (the up element, promoter region, transcriptional start site and cold box region), but excluded the putative translational enhancer region; the downstream box. The ribosome binding site and translational start site were derived from the *lacZ* gene. Expression of this fusion was induced approximately 8-fold over a 5 hour period, when the culture (in exponential phase) was shifted from 37°C to 15°C (figure 4.9). This level of expression was less than predicted from some previous reports of *E. coli* (Lee *et al.*, 1994) and *B. subtilis* (Willimsky *et al.*, 1992) *csp-lacZ* fusions and contrasts with 2D PAGE analysis, which showed that CspA was induced almost 200-fold after

incubation of 1.5 hours at 10°C (figure 6.2). However, the level of expression of *S. typhimurium cspA* may be explained by the absence of some of the putative regulatory elements.

Expression from an *E. coli cspA-lacZ* fusion was increased approximately 18-fold after incubation at 15°C by inclusion of the putative DB (Lee *et al.*, 1994). The corresponding region of DNA that contains the DB is identical in *S. typhimurium*, and was lacking in the *S. typhimurium cspA-lacZ* construct. Thus, it would be reasonable to suggest that the downstream box may be partly responsible for the lower level of expression observed with the *cspA-lacZ* fusion reported in this thesis.

Another possibility for the low level of expression from the *S. typhimurium cspA-lacZ* fusion is that a *cis* acting element (or elements) that is required for normal expression of *cspA* was missing in the plasmid-based construct. Interestingly, studies with *E. coli cspA* have shown that expression from a *cspA-lacZ* fusion that included the putative downstream box, where the reporter gene was fused to codon 62 of *cspA*, only increased 3-fold following incubation for 4 hours at 15°C (Goldenberg *et al.*, 1996). In this case the *cspA-lacZ* construct was inserted into the λ phage attachment site of *E. coli*, which was remote from the native *cspA* locus. A *cspB-lacZ* fusion has also been studied in *B. subtilis* (Willimsky *et al.*, 1992). This construct was also inserted into a phage attachment site on the chromosome and incubation at 15°C did not result in any expression from *cspB-lacZ*. However, when the *B. subtilis cspB-lacZ* fusion was inserted into the chromosome immediately downstream of the native *cspB* gene (by a single downstream cross-over event), expression from *cspB-lacZ* increased approximately 8-fold, following incubation for 2 hours at 15°C (Willimsky *et al.*, 1992). It should be noted that the *B. subtilis cspB-lacZ* fusion did not include any of the *cspB* ORF (*lacZ* was fused immediately downstream of the ribosome binding site), although there does not appear to be a putative downstream box in *B. subtilis cspB* DNA. Both these sets of data indicate that a *cis* acting element (or elements), other than the downstream box, may be necessary for normal cold-induced expression of *E. coli cspA* and *B. subtilis cspB*. In order to gain a clearer understanding of the regulation of *S. typhimurium cspA*, it would be

necessary to assemble a series of reporter constructs, each harbouring different putative regulatory elements.

Regulation of expression of *S. typhimurium cspB* was also investigated using a reporter system. In this case, a bioluminescent reporter element (Mu*dlux*) was inserted into the 23rd codon of the open reading frame of the native, chromosomal *cspB* gene. Expression of *cspB* was not observed at 30°C but was found to increase rapidly, and to a high level, following a temperature shift from 30°C to 10°C, when the culture was in late exponential phase of growth (figure 4.11).

The molecular basis of the cold shock response has not been well studied at temperatures that are below the minimum permissible for growth. However, survival of pathogens such as *Salmonella* and *Listeria* at low temperatures underpins their potential to cause food-borne disease. Thus, gene expression of *S. typhimurium cspB* was investigated at 4°C. Importantly, bioluminescence reporter studies with *cspB* showed that this gene was also induced at 4°C, although the maximum observed level of induction was more than 10-fold less than that observed at 10°C and was delayed by several hours (figure 4.12). Studies with exponentially growing *E. coli* have shown that following a shift from 37°C to 5°C, 70S ribosome sub-units accumulate and translation initiation is blocked (Broeze *et al.*, 1978). This may explain the delay in *S. typhimurium cspB* expression, which appears to be overcome after approximately 12 hours at 4°C.

The influence of global regulators on *cspB* expression at low temperatures were examined. Although σ^s has been reported to be induced in various stress responses (Loewen & Hengge-Aronis, 1994, Hengge-Aronis, 1996) and may even play a regulatory role in some cases, it did not appear to play a major role in the expression of *cspB* at low temperatures. Expression of *cspB* was examined in stationary phase cultures of *S. typhimurium* and found to be induced following a shift from 30°C to 10°C, although induction was delayed relative to that observed when the cultures were in late exponential phase (figure 4.14). Furthermore, *cspB* induction at 4°C was barely detected and further delayed relative to that in the culture at 10°C (figure

4.15). However, Fis was shown to positively affect *cspB* expression when the cells were in stationary phase (figure 4.14). This is surprising in light of the fact that the level of Fis protein has been reported to be very low in stationary phase *S. typhimurium* cells (Osuna *et al.*, 1995). However, Fis has been shown to play a role in the regulation of several genes, measured from *lacZ* fusion constructs, in both late exponential and stationary phase (Xu & Johnson, 1995). Thus, it is possible that Fis affected expression of *cspB* although the cells were in stationary phase.

7.1.3 Protein expression

Previous studies with mesophiles, such as *E. coli*, have shown that the cold shock response of exponentially growing cells at 10°C consists of 2 distinct stages (Jones *et al.*, 1987; Graumann *et al.*, 1996). The initial, adaptive stage involves transient synthesis of a small number of cold-induced proteins (CIPs), including members of the CspA family. The second, acclimation phase involves the resumption of synthesis of the majority of proteins, together with induction of a second set of cold acclimation proteins (CAPs).

Proteins homologues to CspA are found widely in prokaryotes and, to date, more than 50 have been identified in thermophilic, mesophilic, psychrophilic and psychrotrophic bacteria. Furthermore, eukaryotic Y-box proteins, including human YB1, contain a region, termed the 'cold shock domain', that shares 43 % amino acid identity to *E. coli* CspA. Thus, CspA-like proteins are evolutionary highly conserved and probably share common functions. Many bacteria posses several CspA homologues, although they are not all cold shock-inducible. For example, *E. coli* has 9 CspA paralogues of which 4 are induced at low temperature (for a recent review see Yamanaka *et al.*, 1998) *S. typhimurium* contains at least 4 homologues; CspA (Craig & Gallagher, unpublished), CspB (Craig, 1998), CspC (EMBL accession number Af052580) and CspH (EMBL accession number Af006035), of which CspA and CspB are known to be cold-induced (this thesis; Craig *et al.*, 1998). It has been proposed that presence of several CspA-like proteins in *E. coli* has arisen through a

number of gene duplication events (Yamanaka *et al.*, 1998). This has resulted in a family of proteins that have diverse regulation and function.

2- dimensional PAGE has been used to examine *de novo* protein synthesis of *S. typhimurium* at low temperatures, in this thesis. Shifting cultures of exponentially growing *S. typhimurium* from 37°C to 10°C resulted in a distinct pattern of protein expression, typical of many stress responses. Similar to reports for *E. coli* (Jones *et al.*, 1987), a period of adaptation occurred in which overall protein synthesis declined whilst at the same time 14 proteins were induced between 2 and 10-fold, and 6 proteins were induced more than 10-fold (figure 6.2). Amongst these proteins, CspA was the most highly expressed protein and was induced approximately 200-fold. (The level of CspA was barely detectable at 37°C in *S. typhimurium*). After prolonged incubation at 10°C, overall protein synthesis increased so that the number of proteins detected was similar to that at 37°C (figure 6.4). A second set of 28 proteins was induced at this stage, relative to the level detected at 37°C. Furthermore, CspA was induced approximately 20-fold relative to that at 37°C. Thus, *de novo* protein synthesis of *S. typhimurium* is similar to that observed for *E. coli*, in terms of overall protein synthesis and induction of the major cold shock protein (Jones *et al.*, 1987).

Protein expression at 10°C was also examined in a *S. typhimurium* strain that contained a *cspB* mutation (strain MPG361). Several of the proteins that had been observed at a high level during the adaptation phase in the wild type cells were found to be induced during the acclimation phase in the *cspB*$^-$ cells. Furthermore, CspA appeared to be synthesised to a higher level during the acclimation phase in the absence of CspB, relative to that observed in the wild type cells. Thus, mutation of CspB appeared to alter the dynamics of protein expression and the level of CspA, at 10°C.

De novo protein expression was also examined following a temperature downshift from 37°C to 4°C. This was considered important because there are very few reports of the cold shock response at temperatures which are not permissible for growth

(Etchegaray *et al.*, 1996), and the food industry commonly uses 4°C for refrigeration. When exponentially growing cultures of *S. typhimurium* were shifted from 37°C to 4°C, the pattern of protein synthesis was similar to that observed when the culture was shifted to 10°C, but with 2 main differences. Firstly, the timing of the response was delayed in comparison to the response at 10°C and the recovery of overall protein synthesis occurred far later. Secondly, the recovery of overall protein synthesis was not as extensive as that seen at 10°C, even after 4 days at 4°C, where the number of proteins that were detected was only 50% of that observed at 37°C (figure 6.8).

Importantly, these studies show that *de novo* protein synthesis occurs below the minimum temperature permissible for growth and that the block in translation initiation is clearly overcome after extended incubation at 4°C. This indicates that *S. typhimurium* cells are metabolically active in conditions that are normally used to store food. In addition, differences in the dynamics of the cold shock response of *S. typhimurium* occur at 10°C and 4°C, which presumably relate to the ability to grow and divide at the higher temperature.

7.1.4 The effect of growth phase on the cold shock response.

There are few reports of the cold shock response of Gram negative mesophiles in stationary phase. Thus, *de novo* protein synthesis of stationary phase *S. typhimurium* cells was investigated at 10°C and 4°C, which are just above and below the minimum temperature permissible for growth, respectively, using 2-D PAGE. The dynamics of the cold shock response when the cells were in stationary phase were unlike that observed for exponential phase cultures. Overall protein synthesis declined over the period of the experiment, at both 10°C and 4°C. A small number of proteins were induced following the shift to low temperatures and, in particular the synthesis of CspA increased markedly (figure 6.13). CspA was synthesised continually at 10°C and 4°C in stationary phase cultures over the period studied, although the level of synthesis did decrease with time. Furthermore, after 4 days at 4°C, CspA was the only protein that was detected undergoing synthesis (figure 6.20).

Recent studies indicate that the *E. coli* CspA protein is expressed at high levels at 37°C, during early exponential phase (Brandi *et al.*, 1999). The level of the *cspA* transcript was substantial during lag phase and declined rapidly as growth proceeded. In addition, the levels of CspA protein, detected by Western blotting, indicated that substantial amounts of protein were present during lag phase. The extent of cold-shock induction of *cspA* has been proposed to vary with changing growth phase, so that *cspA* gene expression is inversely proportional to the pre-existing level of CspA protein (Brandi *et al.*, 1999). It should be noted that although efforts were made to ensure that only *cspA* mRNA was detected during these experiments, the situation is less clear with regards to the CspA protein. In an attempt to resolve CspA from homologous proteins, non-denaturing electrophoresis was carried out under various conditions of pH and in the presence or absence of urea, prior to hybridisation with a CspA antibody. However, there is a possibility that the CspA-antibody may have cross-reacted with other homologues of a similar molecular size that were present in the whole cell extracts.

The presence of *E. coli* CspA at 37°C, during the lag period may be attributable to ribosome stalling. This has been shown to lead to stabilisation of some mRNAs (Lopez *et al.*, 1998). Moreover, *E. coli* CspA, CspB and CspG have been shown to be synthesised at 15°C in the presence of inhibitors of protein synthesis or following amino acid starvation (Etchegaray & Inouye, 1999). In this case, exponentially growing cells were either incubated in the presence of 0.2 mg ml^{-1} of chloramphenicol or 0.1 mg ml^{-1} of kanamycin at 37°C for 10 minutes prior to a temperature downshift, or were incubated in the absence tryptophan, leucine and methionine at 37°C for 10 minutes prior to the temperature downshift.

Previous studies have suggested that ribosome may act as a temperature sensor where the physical status of the ribosome, *per se*, or some of its products, provide a signal linking the current temperature to the cellular response (VanBogelen & Neidhardt, 1990). In line with this, Jones and colleagues (1992) have suggested that a sharp downshift in temperature induces a physiological state where the translational capacity of the cell is insufficient relative to the supply of charged

tRNAs. This then signals a decrease in the level of (p)ppGpp and the induction of the cold shock response. Taken together, these results suggest that *E. coli* CspA is highly induced, in a temperature-independent mechanism, during a lag in growth, when the translational capacity of the cell has stalled. This hypothesis goes some way to explaining (i) the high level of *S. typhimurium* CspA in exponentially growing cells, during the adaptation phase, at 10°C or 4°C, and (ii) the continuous synthesis of CspA, in stationary phase cells, at 10°C or 4°C.

The effect of growth phase was also investigated on cell survival (measured by colony forming ability on nutrient agar plates). Cultures of *S. typhimurium* were grown at 37°C and at sequential time points samples were diluted into a buffer at 4°C. These studies showed that cells that were in lag phase, late exponential phase and stationary phase were almost completely resistant to the sudden chilling. However, cell survival was only 4 % when the bacteria were in early exponential phase (figure 5.1). The effect of σ^s was assessed during these studies, since this sigma factor is necessary for the regulation of more than 30 stationary phase genes and has also been shown to play a role in stress response regulation in exponential phase (for reviews, see Kolter *et al.*, 1993; Hengge-Aronis, 1996). σ^s did not appear to play a role in resistance to rapid chilling when the cells were in stationary phase, lag phase or early exponential phase. Mutation of the *rpoS* gene led to a marginal decrease in the percentage of bacteria that survived rapid chilling when the culture was in late exponential phase, relative to that observed for the wild type cells (figure 5.3). However, as the decrease was relatively small, it seemed that σ^s did not seem to play a major role in terms of cell survival, at 4°C, under the conditions examined.

The results from the rapid chilling assays, together with the results from *cspB* expression at low temperatures and 2-D PAGE analysis of stationary phase SL1344 cells at low temperatures, indicate that σ^s does not play a major role in regulation of the cold shock response of *S. typhimurium*. This differs from its reported regulatory role in other stress responses (Hengge Aronis, 1996).

Rapid chilling studies were also used to assess the role of osmprotectants on survival in the cold. Previous studies have suggested that *E. coli* bacteria that are maintained in a buffer containing sucrose are resistant to the effects of a sudden shift to 4°C. Cultures of *S. typhimurium* were similarly grown at 37°C and diluted into buffer at 4°C. Addition of sucrose appeared to increase survival of cells that were in early exponential phase (figure 5.2). However, survival of bacteria in late exponential phase approximated that when the cells were cold shocked without the addition of sucrose in the buffer. Thus, osmoprotectants seem to play a small role, increasing the survival of early exponential phase *S. typhimurium* cells at low temperatures. This may have important implications for the food industry where some foods are preserved by addition of these substances.

7.2 FUTURE STUDIES

This study has resulted in characterisation of several aspects of the cold shock response of *S. typhimurium*. Moreover, the information generated has raised more questions concerning the response, some of which are suggested below.

Due to the high level of evolutionary relatedness of *S. typhimurium* and *E. coli* and the high number of CspA paralogues in *E. coli*, it would not be surprising if further CspA homologues exist in *S. typhimurium*. It would be interesting to determine whether any members of the *S. typhimurium* CspA family, currently known or as yet undiscovered, were induced by stresses other than cold shock. Given the differences in the pathogenicity of both organisms, in terms of invasion and intracellular survival, it would be interesting to determine whether any hitherto unknown CspA homologues were induced during invasion. In particular, the intracellular conditions in which *S. typhimurium* persists, for example inside macrophages, presents a very hostile environment which is known to induce stress proteins (Taylor *et al.*, 1998).

The regulation of cold shock genes is complex, with several factors involved, as reported for *E. coli cspA* specifically. Some important questions remain to be answered in terms of regulation of the cold shock response of *S. typhimurium*. Many of the putative regulatory elements that exist in *E. coli cspA* are also apparent in *S.*

typhimurium cspA. However, it is unknown whether they are required for normal expression of *cspA*. It would be interesting to investigate whether there are differences in the thermoregulation of members of the *S. typhimurium* CspA family and address the mechanisms that underlie such differential stability.

Other factors are also likely to play a role in regulation of the cold shock response. It is interesting that the alternative sigma factor, σ^s, does not appear to be involved significantly in the cold shock response, since it has been implicated in other stress responses, in particular the osmotic shock response (Hengge-Aronis, 1996). Other global regulators may be involved, such as H-NS, which is known to be induced following a temperature downshift from 37°C to 10°C, in exponentially growing *E. coli* (Jones *et al.*, 1987). This is a nucleoid associated protein which has been shown, in *E. coli*, to have pleiotrophic effects on transcription and is able to form homomeric or heteromeric complexes (with a paralogous protein, StpA) with distinct functions (Dorman *et al.*, 1999). It has been suggested that at low temperature CspA enhances transcription of *hns* through interaction with the promoter region (Brandi *et al.*, 1994). Thus, it is possible, following a temperature downshift, that CspA increases the cellular concentration of H-NS during the adaptation phase, which may subsequently play a role in enhancing transcription of CAPs during the acclimation phase.

Further studies have suggested that *E. coli* CspA may also enhance transcription of GyrA, another cold-induced protein, in a similar mechanism to that seen with H-NS (Jones *et al.*, 1992). Thus, it seems that *E. coli* CspA may acts as a transcriptional activator of some proteins at low temperatures. More recently, it has been suggested that *E. coli* CspA is an RNA chaperone, making mis-folded RNA more accessible for degradation by RNases (Jiang *et al.*, 1997). This data is in line with the observations that ribosome stalling leads to stabilisation of some mRNAs (Lopez *et al.*, 1998).

Mutation of some cold shock genes has been carried out in *E. coli* (Bae *et al.*, 1997) and *B. subtilis* (Graumann *et al.*, 1996), which has indicated that these proteins play a major role in adaptation to low temperatures. In the present study, attempts to

mutate *S. typhimurium cspA* by allelic exchange did not result in recovery of any mutants. If mutation of *cspA* were possible, it may be necessary to sequentially mutate any other cold-induced *cspA* homologues in order to construct a strain that lacked all the *cspA* homologous. Previous mutation studies with *B. subtilis* (Graumann *et al.*, 1996) and *E. coli* (Bae *et al.*, 1997) suggest that other cold shock homologues may, in part, compensate for the absence of the major cold shock protein. Such a mutant would provide valuable information on the role of the *cspA* homologues in cell survival at low temperature. In addition, a *cspA*$^-$ mutant would indicate how far-reaching the effect of this protein was on overall protein expression at low temperature.

There is no doubt that 2-D PAGE has proved to be an invaluable technique for the study of stress responses. A relatively new technique that complements proteome analysis is micro-array technology, which offers the opportunity to study whole cell transcription under various conditions. This would give quantitative data on gene transcription under a given condition, and would provide a fuller picture of the stress response. Initial studies have shown that this technology is capable of providing a valuable addition to the study of stress responses and in one case has identified genes that are induced during the heat shock response that were previously not identified using conventional techniques (Richmond *et al.*, 1999).

The food industry frequently uses low temperature as a means of food preservation and storage. We have shown that exponentially growing *S. typhimurium* cells are metabolically active when shifted to 4°C, below the minimum growth temperature. However, a lag in protein synthesis occurs when exponentially growing cells are incubated at either 10°C or 4°C, and that the duration of the lag period is temperature-dependent. This lag period may provide an good opportunity to reduce the numbers of viable bacteria, during a time when translation has stalled.

Bacteria that are in stationary phase have been shown to synthesise only a few proteins at low temperatures, including CspA that was the most highly expressed protein. Thus, if this protein is indeed a key factor in low temperature adaptation,

after a suitable interval of time, these cells have a advantage and would be expected to benefit should a favourable change in conditions arise. Furthermore, stationary phase *S. typhimurium* cells have been shown to be very resistant to rapid chilling, therefore, alternative methods may need to be considered to reduce the number of food-associated bacteria in this phase.

APPENDIX I

REFERENCES

Aldsworth, T.G., Sharman, R.L., Dodd, C.E.R. and Stewart, G.S.A.B. (1998). A competitive microflora increases the resistance of *Salmonella typhimurium* to inimical processes: evidence for the suicide response. *Appl. Envion. Microbiol.* **64**: 1323 - 1327

Altschul, S.F., Warren, G., Miller, W., Meyers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215**:403-10

Altuvia, S, Weinstein-Gischer, D., Zhang, A., Postow, L., and Storz, G. (1997). A small, stable RNA induced by oxidative stress: role as a pleiotrophic regulator and antimutator. *Cell.* **90**: 43 –53

Anonymous (1997a). Press Release WHO/58. *World Health Statistics Quarterly* **50:** 1/2 World Health Organization, Geneva, Switzerland.

Anonymous (1997b). WHO fact sheet **139** World Health Organization, Geneva, Switzerland.

Anonymous. (1992). Foodborne pathogenic microorganisms and natural toxins. U.S. Food and Drug Administration Centre for Food Saftey and Applied Nutrition, Washington, DC, USA.

Archer, D.L., and Young, F.E. (1988). Compentorary issues:diseases with a food vector. *Clin. Mic. Rev.* **1**: 377 - 398

Arrhenius, S. (1889). Uber die reakionsgeschwindigkeit bei der inversion von rohrzucker durch sauren. *Z. Phys. Chem.* **4**: 226 – 248

Atlung, T., Knudsen, K., Heerfordt, L., and Brondsted, L. (1997). Effects and σ^s and the transcriptional activator AppY on induction of the *Escherichia coli hya* and *cbdAB-appA* operons in response to carbon and phophate starvation. *J. Bacteriol.* **179**

Av-Gay, G., Aharonowitz, Y., and Cohen, G. (1992). *Streptomyces* contain a 7.0 kDa cold shock like protein *Nucleic Acids Res.* **20**:5478

Bae, W., Phadtare, S., Severinov, K., and Inouye, M. (1999). Charcterization of *Escherichia coli cspE*, whose product negatively regulates transcription of *cspA*, the gene for the major cold shock protein. *Mol. Micro.* **31**(5): 1429 – 1441

Bae,W., Jones,P.G., and Ionuye, M. (1997). CspA, the major cold shock protein of *Escherichia coli* negatively regulates its own gene expression. *J. Bacteriol.* **179**(22): 7081-7088

Bakau, B. (1993) Regulation of the *Escherichia Coli* heat shock response *Mol. Microbiol.* **9**(4);671 - 680

Ball, C.A, Osuna, R., Ferguson, K.C., and Johnson, R.C. (1992). Dramatic changes in Fis levels upon nutrient upshift in *Escherichia coli. J. Bacteriol.* **174**(24): 8043 - 8056

Ball, A.P., Hopkinson, R.B and Farrell, I.D. (1979). Human botulism caused by *Clostridium botulinum* type E: the Birmingham outbreak. *Quaterly J. Med.* **48**: 473 - 491

Baron, A., Jung, J.U. and Villarejo, M. (1987). Purification and characterisation of a glycinEbetaine binding protein in *Escherichia coli. J. Biol. Chem.* **167**: 433 - 438

Bayles, D. O., Annous, B. A., and Wilkinson, B. J. (1996). Cold stress proteins induced in *Listeria monocytogenes* in response to temperature downshock and growth at low temperature *App. Envr. Microbiol.* **62(3)**: 1116 - 1119

Beach, M.B., and Osuna, R. (1998). Identification and characterisation of the *fis* operon in enteric bacteria. *J. Bacteriol.* **180**(22): 5932 - 5946

Benson, N.R., and Goldman, B.S. (1992). Rapid Mapping in *Salmonella typhimurium* with Mu*d*-P22 Prophages *J. Bacteriol* **174**:1673 - 1681

Berger, F., Morellet, N., Menu, F., and Potier, P. (1996). Cold shock and cold acclimation proteins in the physchrotrophic bacterium *Arthrobacter globiformis* SI55. *J. Bacteriol.* **178(11)**:2999-3007

Birnboim, H.C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acid Res.* **7:** 1513-1523.

Blake, P.A, Weaver, D.J. and Hollis, D.E (1980). Diseases of humans (other than cholera) caused by vibrios. *Ann. Rev. Microbiol.* **34**: 341 - 367

Blaser, M.J. and Newman, L.S. (1982). A review of human salmonellosis: I Infective dose. *Rev. Inf. Dis.* **4**: 1096 - 1106

Bolivar, F., Rodriguez, R.L., Betlach, M.C., and Boyer, H.W. (1977). Construction and characterisation of new cloning vehicles. I. Ampicillin-resistant derivatives of the plasmid pMB9. *Gene.* **2:** 75-81

Bolivar, F. (1978). Construction and characterisation of new cloning vehicles. III. Derivatives of plasmid pBR322 carrying unique *Eco*RI sites for selection of *Eco*RI generated recombinant DNA molecules. *Gene.* **4:** 121-136.

Brandi, A., Pietroni, P., Gualerzi, C. O., Pon, C. L. (1996). Post-transcriptional regulation of CspA expression in *Escherichia coli Mol. Microbiol.* **19(2)**: 231 - 240

Brandi, A., Pon, C. L., and Gualerzi, C. O. (1994). Interaction of the main cold shock protein CS7.4 (CspA) of *E. coli* with the promoter region *hns Biochemie* **76**: 1090 - 1098

Brandi, A., Spurio, R., Gualerzi, C.O., and Pon, C.L. (1999). Massive presence of the *Escherichia coli* 'major cold shock protein' CspA under non-stress conditions. *EMBO J.* **18**(6): 1652 – 1659

Broeze, R. J., Solomon, C. J., and Pope, D. H. (1978). Effects of low temperature on in vivo and in vitro protein synthesis in *E. coli* and *Pseuodomonas fluorescens J. Bacteriol.* **134:** 861 - 874

Burd, C.G., and Dreyfuss, G. (1994). Conserved Structures and Diversity of RNA-binding proteins *Science* **265**:615 - 621

Stanier, R.Y., Adelberg, E.A., Ingraham, J.L. (1984) General Microbiology. Macmillan Press Ltd.

Cash, P. (1997). Analysis of bacterial protein synthesis using 2-dimensional electrophoresis. *Presepct. Prot. Eng.* **97**

Cashel, M., and Rudd, K. E. (1987). The stringent response. p 1410 - 1438 *in* Neidhardt, F. C. *et al* (eds) *Escherichia coli* and *Salmonella typhimurium*; cellular and molecular biology. Am. Soc. Microbiol., Washington, D.C.

Chuang, S-E., Daniels, D.L., Blattner, F.R. (1993). Global Regulation of Gene Expression in *Eshcherichia coli J. Bacteriol.***175**:2026 - 2036

Close,T.J., Rodriguez, R.L. (1982). Construction and characterization of the chlorampenicol-resistance gene cartridge: a new approach to the ttranscriptional mapping of extrachromosomal elements. *Gene* **20**: 305-316

Corbett, J.M., Dunn, M.J., Posch, A., Gorg, A.. (1994). Positional reproducibility of protein spots in two-dimensional polyacrylamide gel electrophoresis using immobilised pH gradient isoelectric focusing in the first dimension: An interlaboratory comparison. *Electrophoresis.* **15**: 1205 – 1211

Corpet, F. (1988). Multiple sequence alignment with hierarchical clustering. *Nucl. Acids Res.*, **16** (22): 10881 – 10890

Craig, J.E., Boyle, D., Francis, K.P., and Gallagher, M.P. (1998). Expression of the cold shock gene, *cspB*, in *Salmonella typhimurium* occurs below a threshold temperature. *Microbiol.* **144**: 697 - 704

Csonka, L. N., and Hanson A. D. (1991). Prokaryotic Osmoregulation: Genetics and Physiology. *Annu. Rev. Microbiol.* **45**: 569 - 606

Didier, D.K., Schiffenbauer, J., Woulfe, S.L., Zaceis, M., and Schwartz, B.D. (1988). Characterisation of the cDNA encoding a protein binding to the major histocompatibility complex class II Y box. *Proc. Natl. Acad Sci USA* **85**:7322 – 7326

Dorman, C.J., Hinton, J.C.D. and A. Free (1999). Domain organisation and oligomerisation amoug H-NS-like nucleoid-associated proteins in bacteria. *TIM.* **7**: 124 – 128

Engerecht, J., Simon, M., Silverman, M. (1985). Measuring Gene Expression with Light *Science* **227**:1345 - 1347

Erickson, J. W., Gross, C. A. (1989). Identification of the σ^s subunit of *E. coli* RNA polymerase; a second alternate s factor involved in high-temperature gene expression. *Genes and Development* **3**: 1462 - 1471

Etchegaray, J-P, and Inouye, M. (1999). CspA, CspB and CspG, major cold shock proteins of *Escherichia coli* are induced at low temperature under conditions that completely block protein synthesis. *J. Bacteriol.* **181**(6): 1827 – 1830

Etchegaray, J-P., Jones, P., and Inouye, M. (1996). Differential thermoregulation of two highly homologous cold-shock genes, *cspA* and *cspB*, of *Escherichia coli. Genes to Cells* **1**:171-178

Fang, L., Hou, Y., and Inouye, M. (1998). Role of the cold box region in the untranslated region of the *cspA* mRNA in its transient expression at low temperature in *Escherichia coli. J. Bacteriol.* **180**(1): 90-95

Fang, L., Jiang, W., Bae, W., Inouye, M. (1997). Promoter independant cold-shock induction of *cspA* and its deprepression at 37°C by mRNA stabilization. *Mol. Microbiol.* **23**(2):355-364

Farber, J.M. and Peterkin, P.I. (1991). *Listeria monocytogenes*, a food-borne pathogen. *Microbiol. Rev.* **55**: 476 - 511

Feng, W., Tejero, R., Zimmerman, D.E., Inouye, M., and Montelione, G.T. (1998). Solution NMR structrue and backbone dynamics of the major cold shock protein (CspA) from *Escherichia coli*: evidence for conformational dynamics in the single stranded RNA binding site. *Biochem.* **37**: 10881 - 10896

Feinberg, A.P., and Vogelstein, B. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132:** 3-6. Addendum. *Anal. Biochem.* **137:** 266-267.

Foran, D.R., and Brown, W.M. (1988). Nucleotide sequence of *luxA* and *luxB* genes of the bioluminescent marine bacterium *Vibrio fischeri. Nucleic Acids Res.* **16**: 777 – 777

Forsberg, J.W., Pavitt, G.D., Higgins, C.F. (1994). Use of transcriptional fusions to monitor gene expression: a cautionary tale. *J. Bacteriol.*, **172**: 771 – 778

Francis, K.P. (1993). PhD Thesis, The University of Edinburgh, Scotland, UK

Francis, K.P., and Stewart, G.S.A.B. (1997). Detection and speciation of bacteria through PCR using universal major cold shock protein primer oligomers. *J. Ind. Microbiol. Biotech.* **19**: 286-293

Fraser, C.M. *et al* (1995). The minimal gene complement of *Mycoplasma genitalium Science* **270**: 397-403

Gentry, D. R., Hernandez, V.J., Nguyen, L H., Jensen, D.B., and Cashel, M. (1993). Synthesis of the stationary phase sigma factor σ^S is positively regulated by ppGpp. *J. Bacteriol.* **175(24)**: 7982 - 7989

Gilbert, R.J. and Taylor, A.J. (1976). *Bacillus cereus* food poisoning. in *Microbiology in Agriculture, Fisheries and Food.* Skinner, F.A. and Carr, J.C. (Eds). Academic Press: London.

Goldenberg, D., Azar, I., and Oppenheim, A. B. (1996). Differential mRNA stability of the *cspA* gene in the cold-shock response of *Escherichia coli Mol. Microbiol.* **19(2)**: 241 - 248

Goldstein, J.F., Pollitt, N.S., and Inouye, M. (1990). Major cold shock protein of *E. coli Proc. Natl. Acad. Sci. USA* **87:** 283 - 287

Goransson, M., Sonden, B., Nilsson, P., Dagberg, B., Forsman, K., Emanuelsson, K., Uhlin, B. E. (1990). Transcriptional silencing and thermoregulation of gene expression in *E. coli Nature* **344**:682 - 685

Gorg, A., Postel, W., Gunther, S., Weser, J. (1985). Improved horizontal two-dimensional electrophoresis with hybrid isoelectric focusing in immobilized pH gradoents in the first dimension and laying-on transfers to the second dimension. *Electrophoresis.* **6**: 599-604

Gounot, A-M., (1991). Bacterial life at low temperature: physiological aspects and biotechnological implcations *J. App. Bacteriol.* **71**: 386 - 397

Grauman, P.L., Marahiel, M.A. (1998). A superfamily of proteins that contain the cold shock domain. *TIBS* **23**: 286 - 290

Graumann, P., and Maraheil, M. A. (1994). The major cold shock protein of *B. subtilis* CspB binds with high affinity to the ATTGG- and CCAAT sequences in single stranded oligonucleotides *FEBS Letters* **338**: 157 - 160

Graumann, P., Schroder, K., Schmid, R., and Marahiel, M. (1996). Cold shock stress-induced proteins in *Bacillus subtilis*. *J. Bacteriol.* **178(15)**: 4611-4619

Graumann,P., and Marahiel, M.A.. (1997). Effects of heterologous expression of CspB, the major cold shock protein of *Bacillus subtilis*, on protein synthesis in *Escherichia coli*. *Mol. Gen. Genet.* **253**: 745-752

Graumann,P., Wendrich, T.M., Weber, M.H.W., Schroder, K., and Marahiel, M.A.. (1997). A family of cold shock proteins in *Bacillus subtilis* is essential for cellular growth and for efficient protein synthesis at optimal and low temperatures. *Mol. Micro.* **25**(4):741-756

Hamilton, C., Marti, A., Washburn, B.K., Babitzke, P., Kushner, S.R. (1989). New method for generating deletions and gene replacements in *Escherichia coli* . *J. Bacteriol.* **171**(9):4617-4622

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166:** 557-580.

Hanash, S.M, Strahler, J.R., Somerlot, L., Postel, W., and Gorg, A. (1987). Two-dimensional electrophoresis with immobilised pH gradients in the first dimension: protein focusing as a function of time. *Electrophoresis* **8**: 229 – 234

Hanna, M.M., and Lui, K. (1998). Nascent RNA in transcriptional complexes interacts with CspE, a small protein in *E. coli* implicated in chromatin condensation. *J. Mol. Biol.* **282**: 227 - 239

Hayes, J.D., Kerr, L.A., and Cronshaw, A.D. (1989). *J. Biochem.* **264**: 437 – 445

Hengge-Aronis, R, and Fischer, D. (1992). Identification and molecular analysis of *glgS*, a novel growth phase-regulated and *rpoS*-dependent gene involved in glycogen synthesis in *Escherichia coli*. *Mol. Microbiol.* **6** : 1877 - 1886

Hengge-Aronis, R. (1993). Survival of hunger and stress: the role of *rpoS* in early stationary phase gene regulation in *E. coli*. *Cell* **72**: 165 – 168

Hengge-Aronis, R. (1996). Back to log phase: σ^S as a global regulator in the osmotic control of gene expression in *Escherichia coli*. *Mol. Micrbiol.* **21**(5): 887 - 893

Herendeen, S. L., VanBogelen, R.A., and Neidhardt, F.C. (1979). Levels of major proteins of *Escherichia coli* during growth at different temperatures. *J. Bacteriol.* **139**: 185 – 194

Hogue, A., Akkina, J., Angulo, F., Johnson, R., Peterson, K., Sainin, P., and Schlosser, W. (1997). Situation assessment of *Salmonella typhimurium* DT104. U.S. Department of Agriculture, Food Saftey and Inspection Service, Washington, DC 20250

Holden, N.J., Gallagher, M.P., and Fox, F. (1999). Rapid detection system for stress responses – use in proteomic analysis. Poster presented at the conference: Analysing Protein Structure and Function for Drug Discovery: Applied Proteomics. January 1999.

Hynes, T.R., and Fox, R.O. (1991). The crystal structure of staphylococcal nuclease refined at 1.7 A resolution. *Prot: Structure, Function, Gen* **10**: 92-105

Isch-Horowicz, D., and Burke, J.F. (1981). Rapid and efficient cosmid cloning. *Nucleic Acid Res.* **9:** 2989-2998.

Jeffreys, A.G., Hak, K.M., Steffan R.J., Foster, J.W., and Bej, A.K. (1998). Growth, survival and characterization of *cspA* in *Salmonella enteritidis* following cold shock. *Curr. Microbiol.* **36**: 29-35

Jenkins, D.E., Chaisson, S.A., and Matin, A. (1990). Starvation-induced cross protection against osmotic challenge in *Escherichia coli. J. Bacteriol.* **172**(5): 2779 – 2781

Jenkins, D.E., Schultz, J.E, and Matin, A. (1988). Starvation-induced cross protection against heat and H_2O_2 challenge in *Escherichia coli. J. Bacteriol.* **170**(9): 3910 - 3914

Jiang, W., Fang, L., and Inouye, M. (1996). The role of the 5'-end untranslated region of the mRNA for CspA, the major cold shock protein for *Escherichia coli* in cold shock adaption. *J. Bacteriol.* **178(6)**: 4919-4925

Jiang, W., Hou, Y., and Inouye, M. (1997). CspA, the major cold shock protein of *Escherichia coli* is an RNA chaperone. *J. Biol. Chem.* **272**(1): 196-202

Jiang, W., Jones, P., and Inouye, M. (1993). Chloramphenicol induces the transcription of the major cold shock gene of *E. coli, cspA J. Bacteriol.* **175**: 5824 - 5828

Jones, P. G., Cashel, M., Glaser, G., and Neidhardt, F. C. (1992). Function of a relaxed-like state following temperature downshifts in *E. coli. J. Bacteriol.* **174**: 3903 - 3914

Jones, P. G., and Inouye, M (1994). The cold shock response - a hot topic. *Mol. Microbiol.* **11** (5): 811 - 818

Jones, P., and Inouye, M. (1996). RbfA, a 30S ribosomal binding factor, is a cold-shock protein whose absence triggers the cold-shock response. *Mol. Microbiol.* **21**(6):1207-1218

Jones, P., Mitta, M., Kim, Y., Jiang, W., and Inouye, M. (1996). Cold shock induces a major ribosomal-associated protein that unwinds double-stranded RNA in *Escherichia coli Proc. Natl. Acad. Sci. USA* **93**: 76 - 80

Jones, P.G., Kras, R., Tafuri, S.R., and Wolffe, A.P. (1992). DNA gyrase, CS7.4, and the cold shock response in *E. coli. J. Bacteriol.* **174**: 5798 - 5802

Jones, P.G., VanBogelen, R.A., and Neidhardt, F.C. (1987). Induction of proteins in response to low temperature in *Escherichia coli. J. Bacteriol* **169(5)**:2093 - 2095

Kalnins, A., Otto, K., Ruether, U., and Mueller-Hill, B. (1988). Sequence of the *lacZ* gene of *Escherichia coli. EMBO J.* **2**: 593 – 597

Kandror, O., and Goldberg, A.L. (1997). Trigger factor isinduced upon cold shock and enhances viability of *Escherichia coli* at low temperatures. *Proc. Natl. Acad. Sci. USA* **94**: 4978-4981

Kim, W.S., and Dunn, N.W. (1997). Identification of a cold shock gene in lactic acid bacteria and the effect of cold shock on cryotolerance. *Curr. Microbiol.* **35**: 59-63

Kiyono, M., Omura, T., Inuzuka, M., Fujimori, H., and Pan-Hou, H. (1997). Nucleotide sequence and expression of the organomercurial-resistance determinants from a *Pseudomonas* K-62 plasmid pMR62. *Gene* **189**: 151 – 157

Klauck, E., Bohringer, J., and Hengge-Aronis, R. (1997). The LysR-like regulator LeuO in *Escherichia coli* is involved in the translational regulation of *rpoS* by affectiong the expression of the small regulatory DsrA-RNA. *Mol. Microbiol.* **25**: 559 – 569

Ko, R., Smith, L.T., Smith, G.M. (1994). Glycine Betaine Confers Enhanced Osmotolerance and Cryotolerance on *Listeria monocytogenes J. Bacteriol.* **176**: 426 - 431

Ko, R. and Smith, L.T. (1999). Identification of an ATP-driven osmoregulated glycine betaine system in *Listeria monocytogenes. Appl. Environ. Microbiol.* **65**: 4040 - 4048

Kogure, K., Simidu, U., Taga, N. (1978). A tentive direct microscopic method for counting living marine bacteria. *Can. J. Microbiol.* **25**: 415-420

Kolter, R., Siegele, D.A., and Tormo, A. (1993). The Stationary-Phase of the Bacterial Life-cycle *Ann. Rev. Microbiol.* **47:** 855-874

Kowarz, L., Coynault, C., Robbe-Saule, V., Norel, F. (1994). The *Salmonella typhimurium katF* (*rpoS*) gene; cloning, nucleotide sequence and regulation of the *spvR* and *spvABCD* virulence plasmid genes *J. Bacteriol.* **176**: 6852 - 6860

Kukral, A.M., Strauch, K.L., Maurer, R.A., and Miller C.G. (1987). Genetic analysis in *Salmonella typhimurium* with a small collection of randomly spaced insertions of transposon Tn*10*Δ16Δ17. *J. Bacteriol.* **169**(5): 1787-1793

La Teana, A., Brandi, A., Falconi, M., Spurio, R., Pon, C.L., and Gualerzi, C.O. (1991). Identification of a cold shock transcriptional enhancer of the *E. coli* gene encoding nucleoid protein H-NS *Proc. Natl. Acad. Sci. USA* **88**: 10907 - 10911

Laddaga, R.A., Chu, L., Misra, T.K., S and ilver, S. (1987). Nucleotide sequence and expression of the mercury-resistance operon from *Staphylococcus aureus* plasmid pI258. *Proc. Natl. Acad. Sci.* **84**: 5106 – 5110

Laemmli, U.K. (1970). *Nature* **227**: 680 - 685

Landini, P., Hajec, L.I., Nguyen, L.H., Burgess, R.R., and Volkert, M.R. (1996). The leucine responive regulatory protein (Lrp) acts as a specific repressor for σ^s dependent transcription of the *Escherichia coli aidB* gene. *Mol. Microbiol.* **20** : 947 - 955

Landsman, D. (1992). RNP-1, an RNA-binding motif is conserved in the DNA-binding cold shock domain *Nucleic Acids Res.* **20**:2861 - 2864

Lange, R., Barth, M., and Hengge-Aronis, R. (1993). Complex transcriptional control of the σ^s - dependent stationary phase induced and osmotically regulated *osmY* (*csi-5*) gene suggests new roles for Lrp, Cyclic AMP (cAMP) receptor protein-cAMP complex, and integration host factor in the stationary phase response of *Escherichia coli. J. Bacteriol.* **175(24)**:7910-7917

Lange, R., Fischer, D., and Hengge-Aronis, R. (1995). Identification of Transcriptional Start Sites and the Role of ppGpp in the Expression of *rpoS*, the Structural Gene for the σs Subunit of RNA Polymerease in *E. coli. J. Bacteriol.* **177**: 4676 - 4680

Lange, R., and Hengge-Aronis, R. (1991). Identification of a central regulator of stationary-phase gene expression in *E. coli. Mol. Microbiol.* **5**(1): 49 - 59

Lange, R., Hengge-Aronis, R. (1994). The cellular concentration of the subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation and protein stability. *Genes Dev.* **8**: 1600 – 1612

Lebaron, P., Joux, F. (1994). Flow cytometric analysis of the cellualr DNA content of *Salmonella typhimurium* and *Altermonas haloplanktis* during starvation and recovery in seawater. *Appl. Environ. Microbiol.* **60**(12): 4345-4350

Leboullenger, C., Panoff, J-M. (1996). A bibliography of the cold shock response in prokaryotes. *Cryo-lett.* **17**: 53 - 62

Lederberg, J. (1998). Emerging infections: an evolutionary perpective. *Emerging Infectious Diseases* **4**(3): 366 - 371

Lee, I. S., Lin, J., Hall, H. K., Bearson, B., and Foster, J.,W. (1995). The stationary phase sigma factor σ^s (RpoS) is required for a sustained acid tolerance response in virulent *S. typhimurium. Mol. Microbiol.* **17**(1): 155 - 167

Lee, S. J., Xie, A., Jiang, W., Etchegaray, J., Jones, P.G., and Inouye, M. (1994). Family of the major cold shock protein, CspA (CS7.4), of *E. coli*, whose members show a high sequence similarity with the eukaryotic Y-box binding proteins. *Mol. Microbiol.* **11** (5): 833 - 839

Leilivelt, M.J., and Kawula, T.H. (1995). Hsc66, an Hsp70 homologue in *Escherichia coli* is induced by cold shock but not by heat shock. *J. Bacteriol.* **177**(17): 4900-4907

Loewen, P. (1996). Probing the structure of catalase HPII of *Escherichia coli* – a review. *Gene* **179**: 39-44

Loewen, P.C., and Hengge-Aronis, R. (1994). The role of the sigma factor σ^S (KatF) in bacterial global regulation *Annu. Rev. Microbiol.* **48**: 53 - 80

Loewen, P.C., Hu, B., Strutinsky, J., and Sparling, R. (1998). Regulation in the *rpoS* regulon of *Escherichia coli. Can. J. Microbiol.* **44**: 707-717

Lopez, P.J., Marchand, I., Yarchuk, O., Dreyfus, M. (1998). Translation inhibitors stabilise *Escherichia coli* mRNAs independently of ribosome function. *Proc. Natl. Acad. Sci. USA* **95**: 6067 - 6072

Lopilato, J., Bortner, S. and J. Beckwith. (1986). Mutations in a new chromosomal gene of *Escherichia colii* K-12, *pcnB*, reduce plasmid copy number of pBR322 and its derivatives. *Mol. Gen Genet* **205**: 285 – 290

McGovern, V.P. and Oliver, J.D. (1995). Induction of cold-responsive proteins in *Vibrio vulnificus. J. Bacteriol.* **177**: 4131 - 4133

Marschall, C., Labrousse, V., Kreimer, M., Weichart, D., Kolb, A., and Hengge-Aronis, R. (1998). Molecular analysis of the regulation of *csiD*, a carbon starvation-inducible gene in *Escherichia coli* that is exclusively dependant on σ^s and requires activation by cAMP-CRP. *J. Mol. Biol.* **276**: 339 - 353

Masters, M., Colloms, M.D., Oliver, I.R., He, L., Macnaughton, E.J. and Charters, Y. (1993). The *pcnB* gene of *Escherichia coli*, which is required for ColE1 copy number maintenance, is dispensible. *J. Bacteriol* **175**: 4405 - 4413

Maurice, J. (1994). The rise and rise of food poisioning. *New Scientist* **17 Dec 1994:** 28-33

Mayo, B., Derzelle, S., Fernandez, M., Leonard, C., Ferain, T., Hols, P., Suarez, J.E., and Delcour, J. (1997). Cloning and characterization of *cspL* and *cspP*, two cold shock inducible genes from *Lactobacillus plantarum*. *J. Bacteriol.* **179**(9): 3039-3042

Mayr, B., Kaplan, T., Lechner, S., Scherer, S. (1996). Identification and purification of a family of dimeric major cold shock protein homologs from the phschrotrophic *Bacillus cereus* WSBC 10201 *J. Bacteriol.* **178(10)**: 2916 - 2925

Meighen, E.A. (1991). Molecular biology of bacterial luminescence. *Microbiol. Rev.* **55**(1): 123-142

Meynell, G.G. (1958). The Effect of Sudden Chilling On *E. Coli*. *J. gen. Microbiol.* **19:** 380 - 389

Miller, J.H., (1972). Experiments in molecular genetics. Cold Spring Harbour, New York: Cold Spring Harbour Laboratory Press.

Mitta,M., Fang,L., and Inouye,M. (1997). Deletion analysis of *cspA* of *Escherichia coli*: requirement of the AT- rich UP element for *cspA* transcription and the downstream box in the coding region for its cold shock induction. *Mol. Micro.* **26**(2): 321-335

Mizushima, T., Kataoka, K., Yasujuki, O., Inoue, R., and Sekimizu, K. (1997). Increase in negative supercoiling of plasmid DNA in *Escherichia coli* exposed to cold shock. *Mol. Microbiol.* **23**(2):381-386

Morgan, R.W., Christman, M.F., Jacobson, F.S., Storz, G., and Bruce, N.A. (1996). Hydrogen peroxide-inducible protein in *Salmonella typhimurium* overlap with heat shock and other stress proteins. *Proc. Natl. Acad. Sci.* **83**: 8059 - 8063

Mossel, D.A.A., Jansma, M., and Waart, J.DE. (1981) Growth potential of 114 strain of epidemioloically most common Salmonellae and Arizonae between 3 and 17°C. Chpt. 3, pp.29-39, in *Psychrotrophic micro-organisms in spoilage and pathogenicity*.

Muffler, A., Trasulsen, D. D., Lange, R., Hengge-Aronis, R. (1996). Posttranscriptional osmotic regulation of the σ^S subunit of RNA polymerase in *Escherichia coli* . *Bacteriol* **178(6)**: 1607 - 1613

Muffler, A., Traulsen, D. D., Fischer, D., Lange, R., and Hengge-Aronis, R. (1997). The RNA-binding protein HF-1 plays a global role which is largely, but not exclusively, due to its role in expression of the σ^s subunit of RNA polymerase in *Escherichia coli*. *J. Baceriol.* **179** : 297 – 300

Muffler,A., Barth,M., Marchall,C., and Hengge-Aronis,R. (1997). Heat shock regulation of σ^S turnover: a role for DnaK and relationship between stress responses mediated by σ^S and σ^{32} in *Escherichia coli*. *J. Bacteriol.* **179**(2): 445-452

Mulvey, M.R., and Loewen. (1989). Nucleotide sequence of *katF* of *Escherichia coli* suggests that KatF protein is a novel σ transcription factor. *Nucleic Acids Res.* **17**: 9979 - 9991

Munro, P. M., Flatau, G. N., Clement, R. L., Gauthier, M. J. (1995). Influence of the RpoS (KatF) sigma factor on maintaenacne of viability and cultrability of *Escherichia coli* and *Salmonella typhimurium* in seawater *App. Envr. Microbiol.* **61(5)**: 1853 - 1858

Nakashima, K., Kanamaru, K., Mizuno, T., and Horikoshi, K. (1996). A novel member of the *cspA* family of genes that is induced by cold shock in *Escherichia coli J. Bacteriol.* **178(10)**: 2994 - 2997

Neidhardt, F. C., and VanBogelen, R. A. (1987). The heat shock response. p1334 - 1343 In *Escherichia coli* and *Salmonella typhimurium*; cellular and molecular biology. Neidhardt, F. C. *et al* (eds). Am. Soc. Microbiol., Washington, D.C. UPDATE from new edition

Neidhardt, F., Vaughn, V., Philips, T.A., Bloch, P.L. (1983). Gene-protein index of *Escherichia coli*. *Microbiol. Rev.* **47**(2): 231-284

Newkirk, K., Feng, W., Jiang, W., Tejero, R., Emerson, S. D., Inouye, M., and Montelione, G. T., (1994). Solution NMR structure of the major cold shock protein (CspA) from *Escherichia coli*; identification of a binding eptitope for DNA *Proc. Natl. Acad. Sci. USA* **91**: 5114 - 5118

Ninnemann, O., Koch, C., and Kahmann, R. (1992). The *E. coli fis* promoter is subject to stringent control and autoregulation. *J. EMBO* **11**: 1075 - 1083

Nystrom, T. Neidhardt, F. (1996). Effects of overproducing the universal stress protein, UspA, in *Escherichia coli*. *J. Bacteriol.* **178**: 927 – 930

Nystrom, T., and Neidhardt, F.C. (1992). Cloning, mapping and nucleotide sequence of a gene encoding a universal stress protein in *Eshcherichia coli Mol. Microbiol.* **6**(21): 3187 - 3198

O'Farrell, P. (1975). High resolution of two-dimensional electrophoresis of proteins. *J. Bio. Chem.* **250(10)**:4007-4021.

Orskov, F., Orskv, I. and Villon, J.A. (1987). Cattle as a reservoir of verotoxin-producing *Escherichia coli* O157:H7. *Lancet II*, 276

Oshima, T., Aiba, H., Baba, T., Fujita, K., Hayashi, K., Honjo, A., Ikemoto, K., Inada, T., Itoh, T., Kajihara, M., Kanai, K., Kashimoto, K., Kimura, S., Kitagawa, M., Makino, K., Masuda, S., Miki, T., Mizobuchi, K., Mori, H., Motomura, K., Nakamura, Y., Nashimoto, H., Nisho, Y., Saito, N., Sampei, G., Seki, Y., Tagami, H., Takemoto, K., Wada, C., Yamamoto, Y., Yano., M., and Horiuchi, T. (1996). A 718-kb DNA sequence of the *Escherichia coli* K-12 genome corresponding to the 12.7 – 28.0 minute region on the linkage map. *DNA Res.* **3**: 137 – 155

Osuna, R., Lienau, D., Hughes, K.T., and Johnson, R.C. (1995). Sequence, regulation and functions of *fis* in *Salmonella typhimurium*. *J. Bacteriol.* **177**(8): 2021-2032

Ozaki, M., Wada, A., Fujita, N., Ishihama, A. (1991). Growth phase-dependant modification of RNA polymerase in *E. coli. Mol. Gen. Genet* **230:** 17 - 23

Pang, T., Zulfiiquar, A.B., Finlay, B.B., and Altwegg, M. (1995). Typhoid fever and other salmonellosis: a continuing challenge. *Trends Microbiol* **3**(7): 253 - 255

Panoff, J-M., Corroler, D., Thammavongs, B., and Boutibonnes, P. (1997). Differentiation between cold shock proteins and cold acclimation proteins in mesophilic grampositive bacterium, *Enterococcus faecalis* JH2-2. *J. Bacteriol.* **179**(13): 4451-4454

Panoff, J-M., Thammavongs, B., Guegen, M., and Boutibonnes, P. (1998). Cold stress responses in mesophilic bacteria. *Cryobiol.* **36**: 75 - 83

Pao, C.C., and Dyess, B.T. (1981). Stringent control of RNA synthesis in the absence of guanosine 5'-triphosphate-3'-diphosphate. *J. Bio. Chem.* **256(5)**: 2252-2257

Pearson, W.R. (1996) *Meth. Enzymol.* **266**:227-258

Phanthanh, L. and Gormon, T. (1995). Analysis of heat and cold shock proteins in *Listeria* by 2-dimensional electrophoresis. *Electrophoresis* **16**: 444 - 450

Qi, S-Y., Moir, A., and O'Connor, C.D. (1996). Proteome of *Salmonella typhimurium* SI1344: identification of novel abundant cell envelope proteins and assigment of a two dimensional reference map. *J. Bacteriol.* **178**(16):5032-5038

Qoronfleeh, M. W., Debouck, C., Keller, J. (1992). Identification and characterization of novel low-temperature-inducible promoters of *E. coli. J. Bacteriol.* **174:** 7902 - 7909

Richmond, C.S., Glasner, J.D., Mau, R., Jin, H., and Blattner, F.R. (1999). Geneome-wide exression profiling in *Escherichia coli. Nuc. Acids Res.* **27**: 3821 – 3835

Rodriguez, G.G., Phillips, KD, Ishiguro, K., Ridgeway, H.F. (1992). Use of a fluorescent redox probe for direct visualisation of actively respiring bacteria. *Appl. Environ. Microbiol.* **58**: 1801 - 1808

Ross, W., Gosink, K.K., Salomon, J., Igarashi, K., Zou, C., Ishihama, K., Sererinov, K., and Grourse, R.L. (1993). A thrid recognition element in bacterial promoters: DNA binding by the α-subunit of RNA polymerase. *Science* **262**: 1407 - 1413

Roszak, D.B., Grimes, D.J., Colwell, R.R. (1983). Viable but nonrecoverable stage of *Salmonella enteritidis* in aquatic systems. *Can. J. Microbiol.* **30**: 334-337

Rowe, B., Gilbert, R.J. and N.T. Begg (1987). *Salmonella ealing* infections associated with consumption of infant dried milk. *Lancet.* **11**: 900 - 903

Russell, C.B., Thaler, D.S., and Dahlquist, F.W. (1989). Chromosomal transformation of *Escherichia coli recD* strains with linearised plasmids. *J. Bacteriol.* **171**(5): 2609 - 2613

Ryan, C.A., Nickels, M.K. and Hargrett-Bean, N.T. (1987). Massive outbreak of antimicrobial-resistant salmonellosis traced to pasturised milk. *J. Am. Med. Assoc.* **258**: 3269 – 3274

Sato, N. and Murata, N. (1981). Studies on the temperature shift-induced desaturation of fatty acids in monogalactisyl diacylglycerol in the blue-green algae (cyanobacteria) *Anabaena variabilis. Plant cell Physiol.* **22**: 1043 - 1050

Schagger,H., and Von Jagow,G. (1987). Tricine - sodium dodecyl sulphate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100kDa. *Anal. Biochem.* **166**: 368-379

Schevchenko, A., Chernushevich, I., Ens, W., Standing, K.G., Thomson, B., Wilm, M., and Mann, M. (1998). Rapid *de novo* peptide sequencing by a combination of nanoelectrospray, isotpic labelling and a quadrupole/time of flight mass spectrmeter. *Rapid Comm. Mass Spec.* **11**: 1015 – 1024

Schindelin, H., Jiang, W., Inouye, M., and Heinmann, U. (1994). Crystal structure of the CspA, the major cold shock protein of *E. coli. Proc. Natl. Acad. Sci. USA* **91**: 5119 - 5123

Schroder, K., Zuber, P., Willimsky, G., Wahner, B., and Maraheil, M. A. (1993). Mapping of the *Bacillus subtilis cspB* gene and cloning of its homologues in thermophilic, mesophilic and pyschrotrophic bacilli *Gene* **136**: 277 - 280

Schweder, T., Lee K-H., Lomosvaya, O, and Martin, A. (1996). Regulation of *Escherichia coli* Starvation sigma factor (σ^s) by ClpXP protease. *J. Bacteriol.* **178**(2): 470 - 476

Sharman, and Cameron (1934). Lethal environmental factors within the natural range of growth. *J. Bacteriol.* **27**: 341

Shaw, W.V. (1975) Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria, in Colowick, S.P. and Kaplan, N.O. (Eds), *Methods in Enzymology.* Academic Press, New York, 735-755

Short, J.M., Fernandez, J.M., Sorge, J.A, and Huse, W.D. (1988). λZAP: a bacteriophage λ expression vector with *in vivo* excision properties. *Nucleic Acid Res.* **16:** 7583-7600.

Silhavy, T.J., Berman, M.L., and Enquist, L.W. (1984). DNA extraction from bacterial cells. In *Experiments with gene fusions*. pp137 – 140. Cold Spring Harbour, New York: Cold Spring Harbour Press.

Silver, S. and Walderhaug, M. (1992). Gene regulation of plasmid-and chromosome-determined inorganic ion transport in bacteria. *Microbiol. Rev.* **56**: 195 - 228

Sledjeski, D.D., Gupta, A., and Gottesman, S. (1996). Then small RNA, DsrA, is essential for low temperature expression of RpoS during exponential growth in *Escherichia coli. EMBO* **15**(15): 3993-4000

Smith, J. J., Howington, J. P., and McFeters, G. A. (1994). Survival, physiological response, and recovery of enteric bacteria exposed to a polar marine environment *App. Env. Microbiol.* **60(8)**; 2977 - 2984

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98:** 503-517.

Spector, M.P., Aliabadi, Z., Gonzalez, T., and Foster, J.W. (1986). Global control in *Salmonella typhimurium*: two-dimensional electrophoretic analysis of starvation-, anaerobiosis-, and heat-shock inducible proteins. *J. Bacteriol.* **168**(1): 420 - 424

Spector, M.P., and Cubitt, C.L., (1992). Starvation-inducible loci of *Salmonella typhimurium*: regulation and roles in starvation-survival. *Mol. Microbiol.* **6**(11): 1467 - 1476

Sprengart, M.L., Fuchs, E. and Porter, A.G. (1996). The downstream box: an efficient and independent translation initiation signal in *Escherichia coli*. *EMBO J.* **15**: 665 – 674

Stanier, R.Y., Adelberg, E.A. and Ingraham, J.L. (1984). General Microbiology.The MacMillan Press Ltd., London.

Tanabe, H., Goldstein, J., Yang, M., and Inouye, M. (1992). Identification of the promoter region of the *E. coli* major cold shock gene, *cspA* *J Bacteriol.* **174**: 3867 - 3873

Tartaglia, L.A., Storz, G., Brodsky, M.H., Lai, A. and Ames, B.N. (1990). Alkyl hydroperoxide reductase from *Salmonella typhimurium*. *J. Biol. Chem.* **265:** 10535-10540.

Taylor, P.D, Inchley, C.J. and Gallagher, M.P. (1998). The *Salmonella typhimurium* AhpC polypeptide is not essential for virulence in BALBB/c mice but is recognised as an antigen during infection. *Inf. Imm.* **66**: 3208 - 3217

Tomb, J-F, White, O., Keriavage, A.R., Clayton, R., Sutton, G.G., Flleischmann, R.D., Ketchum, K.A., Klenk, H.P., Gill, S., Dougherty, B.A., Nelson, K., Quackenbush, J., Zhou, L., Kirkness, E.F., Peterson, S., Loftus, B., Richardson, D., Dodson, R., Khalak, H.G., Glodek, A., McKenny, K., Fitzgerald, L.M., Lee, N., Adams, M.D., Hickey, E.K., Berg, D.E., Gocayne, J.D., Utterback, T.R., Peterson, J.D., Kelley, J.M., Cotton, M.D., Weldman, J.M., Fujii, C., Bowman, C., Watthey, L., Wallin, E., Hayes, W.S., Borodovsky, M., Karp, P.D., Smith, H, O., Fraser, C.M., and Venter, J.C. (1997). The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* **388**: 539 - 547

US Food and Drink Administration Centre for Food Saftey and Applied Nutrition. (1992). Foodborne Pathogenic and their natural toxins. (Bad Bug Book).

Van Dyk, T.K., Smulski, D.R., Reed, T.R., Belkin, S., Vollmer, A.C., and LaRossa, R.A. (1995). Responses to toxicants of an *Escherichia coli* strain carrying a *uspA'::lux* genetic fusion and an *E. coli* strain carrying a *grpE'::lux* fusion are similar. *Appl. Environ. Microbiol.* **61**(11): 4124 - 4127

VanBogelen, R. A., and Neidhardt, F. C. (1990). Ribosomes as sensors of heat and cold shock in *E. coli*. *Proc. Natl. Acad. Sci. USA* **87:** 5589 - 5593

VanBogelen, R., Abshire, K.Z., Pertsemlidis, A., Clark, R.I., and Neidhart, F.C. (1996) Gene-Protein Index of *E. coli* K-12, edition 6. p. 2067 –2117 *in* Neidhardt, F. C. *et al* (eds) *Escherichia coli* and *Salmonella typhimurium*; cellular and molecular biology. Am. Soc. Microbiol., Washington, D.C.

Varnam, A.H., and Evans, M.G. (1996). Salmonella. In *Foodborne pathogens: an illustrated text*. Varnam, A.H., Evans, M.G. (Eds.) Manson Publishing.

Vieira, J., and Messing, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthestic universal primers. *Gene* **19**: 259 - 268

Vigh, L., Maresca, B., and Harwood, J.L. (1998). Does the membrane's physical state control the expression of heat shock and other genes? *TIBS* **23**: 369 - 374

Volker,U., Mach, H., Schmid, R., Hecker, M. (1992). Stress proteins and cross-protection by heat shock and salt stress in *Bacillus subtilis J. Gen Microbiol.* **138**:2125 - 2135

Von Ossowski, I., Mulvey, M.R., Leco, P.A., Borys, A., and Loewen, P.C. (1991) Nucleotide sequence of *Escherichia coli katE*, which encodes catalase HPII. *J. Bacteriol.* **173**(3): 514-520

Wada, H. and Murata, N. (1990). Temperautre-induced changes in the fatty acid composition of the cyanobacterium, *Synechocystis* PCC 6803. *Plant Physiol.* **92**: 1062 - 1069

Wada, H., Gombos, Z., Murata, N. (1990). Enhancement of chilling tolerance of a cyanbacterium by genetic manipulation of fatty acid desaturation *Nature* **347:** 200 - 203

Wallis, T.S., Vaughan, A.T.M., and Clark, G.J. (1990). The role of leukocytes in the induction of fluid secretion by *Salmonella typhimurium. J. Med. Micro.* **31**: 27 - 35

Walker, G. C. (1987). The SOS response of *Escherichia coli*. p. 1346 - 1354 *in* Neidhardt, F. C. *et al* (eds) *Escherichia coli* and *Salmonella typhimurium*; cellular and molecular biology. Am. Soc. Microbiol., Washington, D.C.

Wang, J.C. DNA Topoisomerases (1985). *Ann. Rev. Biochem.* **54**: 665 - 697

Wang, N., Yamanaka, K., and Inouye, M. (1999). CspI, the ninth member of the CspA family of *Escherichia coli* is induced upon cold shock. *J. Bacteriol.* **181**(5): 1603 - 1609

Wilkins, M.R., Gasteiger, E., Tonella, L., Ou, K., Tyler, M., Sanchez, J-C., Gooley, A.A., Walsh, B.J., Bairoch, A., Appel, R.D., Williams, K.L., and Hochstrasser, D.F. (1998). Protein identification with N and C-terminal sequence tags in proteome projects. *J. Mol. Biol.* **278**: 599 – 608

Willimsky, G., Bang, H., Fischer, G., and Maraheil, M.A. (1992). Characterization of *cspB*, a *B. subtilis* inducible cold shock gene affecting cell viabilty at low temperature *J. Bacteriol.* **174**: 6326 - 6335

Wolffe, A.P. (1993). Structural and Functional Properties of the Evolutionarily Ancient Y-box Family of Nucleic Acid Binding Proteins *BioEssays* **16**:245 - 251

Xu, J., and Johnson, R.C. (1995). Identification of genes negatively regulated by Fis: Fis and RpoS co-modulate growth-phase-dependent gene expression in *Escherichia coli. J. Bacteriol.* **177** : 938 – 947

Yamanaka, K., Fang, L., and Inouye, M. (1998). The CspA family in *Escherichia coli* : multiple gene duplication for stress adaptation. *Mol. Micro.* **27**(2): 247-255

Yamanaka, K., and Inouye, M. (1997). Growth phase dependant expression of *cspD*, encoding a member of the CspA family in *Escherichia coli. J. Bacteriol.* **179**(16): 5126-5130

Yamanaka, K., Mitani, T., Ogura, T., Niki, H., and Hiraga, S. (1994). Cloning, sequencing, and characterisation of multicopy supressor of a *mukB* mutation in *Escherichia coli. Mol. Microbiol.* **13(2)**: 301 - 312

Yamanaka, K., Mitta, M., and Inouye, M. (1999). Mutation analysis of the 5' untranslated region of the cold shock *cspA* mRNA of *Escherichia coli J. Bacteriol.* **181**: 6284 – 6291

Yamashino, T., Ueguchi, C., and Mizuno, T. (1995). Quantative control of the stationary phase-specific sigma factor, σ^S, in *E. coli*: involvement of the nucleoid protein H-NS. *The EMBO Jounral* **14**: 594 - 602

Yurieva, O., Kholodii, G., Minakhim, L., Gorlenko, Z., Kalyaeva, E., Mindlin, S., Nikiforov, V. (1997). Intercontinental spread of promiscuous mercury-resistance transposons in environmental bacteria. *Mol. Microbiol.* **24**: 321 - 329

Zubiaga, A.M., Belasco, J.G., Greenberg, M.E. (1995). The nonamer UUAUUUAUU is the key AU-rich seqence motif that mediates mRNA degredation. *Mol. Cell. Biol.* **15**(4): 2219-2230